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"Evaluation of the effects and interactions of gas blending and feeding strategy on a Fab′ fermentation by *Escherichia coli*"

by

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For my dearest family: Flor del Monte, Rubén Joel, and Ribia.

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Contents

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Nomenclature

C_{inlet}	inlet oxygen concentration (mol/L)
C_{outlet}	outlet oxygen concentration (mol/L)
C*	oxygen concentration at equilibrium (mol/L)
D	diameter of impeller (m)
H	Henry's constant (atm.L/moL)
H_L	liquid height (m)
K_La	volumetric mass transfer coefficient (h^{-1})
$n_{impeller}$	impeller number
N	impeller speed (rpm or rps)
V	working volume (L)
Po	power consumption under non-aerated conditions (W)
Pg	power consumption under gassed conditions (W)
P_{tot}	total pressure
Q	volumetric gas flow rate (m^3/s)
Re	Reynold's number
t_C	circulation time (s)
t_m	mixing time (s)
t_{OC}	time for oxygen consumption (s)
t_{OT}	time for oxygen transfer (s)
Vgs	superficial gas velocity (m/s)
CER	carbon dioxide evolution rate ($mmoL/L.h^{-1}$)
DOT	dissolved oxygen tension

DCW	dry cell weight (g/L)
GUR	glycerol uptake rate (g/h)
GB	gas blending
non-GB	non gas blending
OUR	oxygen uptake rate (mmoL/L.h^{-1})
RQ	respiratory quotient
μ_{max}	maximum specific growth rate (h^{-1})
δ	density (kg/m^3)
IPTG	isopropyl-β-D-thiogalactopyranosid
PPG	polypropylene glycol

Abstract

A method to improve production of Fab' fragments using gas blending and pH-stat feeding strategy in a fermentation process with *Escherichia coli* has been developed. Regime analysis together with design of experiments (DoE) has been used to evaluate the effect of gas blending and feeding strategy on a Fab' fermentation process. The economic implications of the fermentation strategy have also been considered.

Batch-feeding fermentations carried out at 20 L and 450 L scale indicated that cascade control was not sufficient to maintain a constant DOT level throughout the fermentation. DOT levels dropped to zero during induction phase at both scales. Regime analysis was performed based on experimental K_La determination. K_La values of ~400 h^{-1} were observed at 20 L and 450 L scale. Comparison of time of oxygen consumption (t_{OC}) and time of oxygen transfer (t_{OT}) suggested that O_2 limitation is present in this fermentation process and that this worsens as the scale increases.

A gas blending system at 20 L scale was proposed to address this problem. A factorial 2^2 experimental design was executed to evaluate independently the effects and interactions of two main engineering factors (related to oxygen transfer in the broth) on Fab' titre: DOT level and agitation rate. Gas blending was successful in maintaining constant levels of DOT at 20 L scale. Fab' production was increased by 77 % at of agitation rate of 500 rpm independent of the DOT level, compared to operation at the same scale but without gas blending. High levels of product localisation in the periplasm of 84 - 93% were also obtained. Furthermore, based on t_{OC} and t_{OT}, it could be suggested that no oxygen limitation is likely to occur in the fermentations performed with gas blending, regardless of the agitation rate.

Batch-fed fermentations either with or without gas blending carried out at 20 L scale indicated the presence of glycerol oscillations. A pH-stat feeding strategy in a gas blending system was implemented to address this problem. Results showed that a 2-fold increase in the production of Fab' at 20 L scale, compared to a fermentation operated in a pulsed fed-batch mode could be achieved. A consistently high level (>90%) of product localisation in the periplasm was also achieved and no negative impact on product recovery was observed.

Finally, a preliminary economic analysis was performed to estimate the production costs in £/mg of product. The effects of gas blending and feeding strategy at 20 L scale on the final product cost were evaluated by comparing: batch-fed non gas-blending, batch-fed gas blending, and pH-stat gas blending fermentations. A fermentation production cost of ~£1.45/mg of Fab' was estimated for a pH-stat gas blending system. This resulted in cost savings of up to 75% when compared to the production cost of ~£5.80/mg of Fab' in the batch-fed system without gas blending.

The results obtained in this work provide an impetus for further studies to evaluate the potential of gas blending and pH-stat feeding strategy for the industrial production of Fab' antibody fragments. The use of statistical design of experiments together with regime analysis was found to be a very useful tool to gain a better understanding of this system

Chapter 1

Antibody Processing

In recent years, a rapid increase in the scientific and commercial interest in antibodies and antibody-based molecules has been observed, and production processes whereby these proteins may be produced efficiently and at low cost are being studied (Harrison et al, 1996). Antibodies represent a very interesting class of proteins, not only because of their capacity to recognise another molecule with high affinity and specificity, but also because of their stability and the possibility to engineer them at will.

Antibodies origins can be found in an elaborate protective set-up known as the immune system (*immunis*, exempt) that animals have evolved in order to deal with disease-causing microorganisms. The immune system is conferred by certain types of white blood cells which are collectively known as lymphocytes and it is divided in two parts:

a. Cellular immunity, which guards against virally infected cells, fungi, parasites, and foreign tissue.
b. Humoral immunity (*humor*, fluid), which is most effective against bacterial infections and the extracellular phases of viral infections. This system is mediated by an enormously diverse collection of related proteins known as antibodies or immunoglobulins.

The immune system response is triggered by the presence of foreign macromolecules, normally proteins, carbohydrates, and nucleic acids, known as antigens. It has the capacity to generate antibodies against almost any antigen that encounters by producing virtually unlimited variety of antigen-binding sites (Voet et al, 1995).

Antibodies can bind virtually any kind or size of molecule (from large proteins to organic ligands) and could replace substances in different applications (e.g. food, cosmetic, chemical and environmental sectors) with undesirable medical, social or environmental side effects (Harris, 1999).

1.1. STRUCTURE & USES OF ANTIBODIES

The basic building block of antibodies ("Y" shaped polypeptide tetramers) consists of four subunits: two identical ~23-kD light chains and two identical 53- to 75-kD heavy chains (Voet et al, 1995). As can be observed in figure 1.1, the amino-terminal end provides variability (V) in both the heavy (H) and the light (L) chains. These variable regions are known as V_H and V_L, respectively, and together they form the antigen-binding site. The remainder of the whole antibody consists of constant domains, where the light chain is known as C_L, and the heavy chain constant section is further divided into three structurally discrete regions C_{H1}, C_{H2}, and C_{H3} (Harrison et al, 1996).

The structure of the antigen-binding site physically matches the antigen like a glove: the antibody's region forms an opening to surround the antigen's protrusion. Hydrogen bonds stabilise the antibody-antigen interaction. In addition to hydrogen bonds, other weak interactions such as van der Waals forces, hydrophobic interactions and electrostatic forces improve the binding specificity between antibody and antigen. These interactions occur over large and sometimes discontinuous regions of the molecules, improving binding affinity. Affinity refers to the strength of binding and, specificity refers to the ability of a protein to bind one molecule in preference to other molecules.

The antibody binding sites bind their targets at essentially a 1:1 molar ratio and so it is unnecessary and uneconomical to use an antibody of molecular weight 150 kDa to bind a target of 200 Da. Consequently, one important aim is to construct the lowest molecular weight fragment that can still bind its antigen with high affinity. The smaller the fragment, the greater the number of binding sites per unit of protein and the lower the unit cost of manufacture (Harris, 1999). In Figure 1.1 a variety of antibody fragment configurations that have been recently developed are shown.

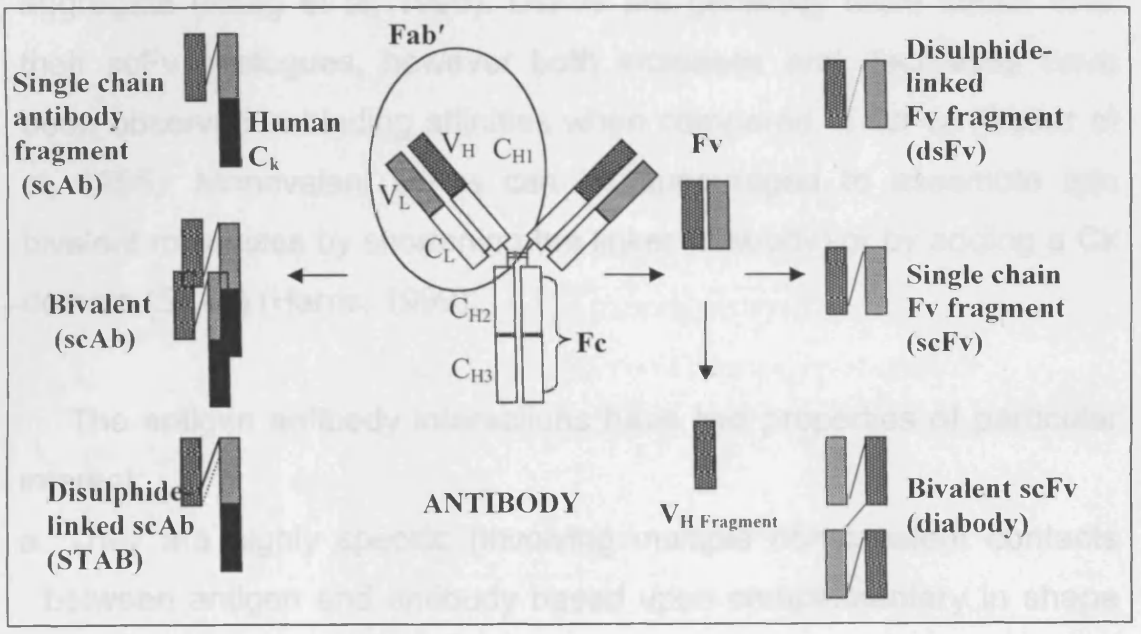


Figure 1.1 Schematic representations of an antibody and different fragment configurations.

The Fv fragment is the smallest antibody fragment that still contains the complete antigen binding site with the same properties as the Fab' fragment, which has been documented as having the same activity as the whole antibody (Skerra et al, 1988). Nevertheless, they are quite unstable and have a tendency to dissociate into V_H and V_L upon dilution (Glockshuber et al, 1990).

In order to increase the stability of the Fv fragment, the single chain Fv fragment and the disulphide stabilized Fv fragment were designed to link the two variable domains. Ideally, the linker should be sufficiently long to span the distance between the two domains, flexible to allow the association and must not affect the antigen binding site (Raag et al, 1995).

Although many scFvs have good binding activities, several show a reduced affinity when compared to the Fab' fragment (Bird et al, 1991) and also have a tendency to form multimers that are unstable and aggregate (Raag et al, 1995). DsFvs are generally more stable than their scFv analogues, however both increases and decreases have been observed in binding affinities when compared to scFvs (Reiter et al, 1996). Monovalent scFvs can be encouraged to assemble into bivalent molecules by shortening the linker (diabody) or by adding a Ck domain (ScAb) (Harris, 1999).

The antigen antibody interactions have two properties of particular interest:

a. They are highly specific (involving multiple non-covalent contacts between antigen and antibody based upon complementary in shape and charge)
b. They have high binding strengths (with affinities between 10^8 and 10^{11} M^{-1}).

The prospect of being able to exploit these properties and generate molecules of high binding affinity against any chosen target, led Paul Ehrlich to predict (in his Croonian Lecture to the Royal Society in 1900) an "age of magic bullets" providing great applications in human therapeutics (Harris, 1999). Table 1.1 shows some of the general and potential uses of antibody fragments in the industrial sector.

Table 1.1 General and potential uses of antibody fragments in industrial Sectors (adapted from Berry et al, 1991; Harris, 1999; Harrison et al, 1996; Sotiriadis et al, 2001)

INDUSTRIAL SECTOR	USE OF ANTIBODY FRAGMENTS
Pharmaceutical	• Therapeutic uses concerned with tumour imaging and therapy. They can easily be attached to radiolabels or toxins to enable imaging and treatments of tumours. In addition, their reduced size allows the penetration of dense tumour tissue. • Immunoaffinity purification columns where the size of antibody fragments allows immobilization to a greater capacity on porous supports. • Enzymes for prodrug therapy, viruses for gene therapy and liposomes for the delivery of drugs, toxins and DNA, and *in vitro* immunoassay assays for clinical diagnosis.
Food	• Inhibiting enzymes that cause food spoilage. • Molecular mimics to mask flavours and odours.
Cosmetics and toiletries	• Antibacterials and antifungals for toothpastes and mouthwashes. • Inhibiting enzymes that cause body odours.
Detergents	• Protecting sensitive motifs during processing. • Stain removal.
Manufacturing industry	• Separating products from intermediates. • Clean-up of process waters.
Environmental protection	• Removing microorganisms and viruses from water. • Removing organic pollutants from water. • Bioremediating land.
Biosensors	• Monitoring: a). Chemical pollution of the environment. b). Microbial contamination. c). Food deterioration.

Such widespread applications require that antibodies or their fragments can be available in large quantities, at an acceptable cost, in configurations with a high affinity for their targets and high stability in adverse environments. In recent years, due to the increasing commercial demand, production processes and their key variables have been studied in different systems (Harris, 1999).

1.2. PRODUCTION OF ANTIBODY FRAGMENTS

Antibody fragments can be produced either by proteolytic digestion of whole antibodies or expressed as recombinant proteins in microorganisms and mammalian cells. However, the first method will usually give a heterogeneous mixture due to non-specific cleavage and differences in the susceptibility of antibodies to protease action (Porter, 1959; Inbar et al, 1972).

On the other hand, the selection of a suitable expression system has a significant effect on the productivity of the process and the complexity of the downstream processing. The principal options to obtain antibody fragments by cell culture include:

a. Mammalian cells, used for the production of monoclonal antibodies.
b. Recombinant microorganisms, such as *Escherichia coli*, *Trichoderma reesei*, *Pichia pastoris*, *Streptomyces lividans*, *Proteus mirabilis*, *Aspergillus awamori* and *Saccharomyces cerevisiae* (Frenken et al, 1998; Sotiriadis et al, 2001).
c. Edible plants, which can be used in purifying the products for human or animal consumption. The ease of purification varies considerably between species and may be related to the different levels of carbohydrates, protein and oil; species such as wheat, potato and pea give relatively easy purification (Harris, 1999). Nevertheless, there is no evidence of large scale production using this kind of technology.

Regarding the manufacturing of antibody fragments by means of bacteria, *E. coli* offers a number of advantages: (Baneyx, 1999; Better, et al, 1989)

a. *E. coli* is well characterised at the molecular level, with well-established techniques for genetic manipulation.
b. The introduction of foreign genes can be achieved relatively easily, with proteins being expressed to high titres at specific locations within the cell.
c. The availability of an increasingly large number of cloning vectors and mutant host strains.
d. The large-scale production of antibody fragments is relatively simple due to its fast growth to high cell density and its fermentation process (using inexpensive media).
e. The successful production of antibody fragments and other recombinant proteins is well documented.

The possibility of producing antibody Fab' fragments in *Escherichia coli* has been a major break-through in the field of antibody engineering (Skerra et al, 1988). Since then, considerable effort has been put into the optimisation of production levels (Nielsen et al, 2003). A disadvantage of *E. coli*, however, is the factor that the cells have to be disrupted in order to release the product and subsequently extensive purification is required (Frenken et al, 1998). Recent work has investigated the production of antibody fragments in different organisms such as filamentous fungus (Sotiriadis et al, 2001).

Many different strategies have been developed in order to achieve not only high quantities of antibody fragments, but also efficient and inexpensive purification.

1.3. EXPRESSION STRATEGIES USING *E. coli*

A major problem in almost every area of biochemical research is obtaining sufficient quantities of the product of interest. In the case of antibody fragments and many other products this has been overcome by methods referred to as genetic engineering or recombinant DNA technology.

Specifically, a cloned structural gene must be inserted into an expression vector, a plasmid (small, autonomously replicating DNA molecule) that contains the properly positioned transcriptional and translational control sequences for the antibody fragment's expression. With the use of a relaxed control plasmid and an efficient promoter (a type of transcriptional control element), the production of the antibody fragment may reach 30% of the *E. coli's* total cellular protein (Voet et al, 1995).

A variety of expression vectors are available commercially for *E. coli* to produce antibody fragments. Most of them use moderate to high copy number of plasmids which can drive rapid protein expression (Swartz, 2001).

However, there is no guarantee that a recombinant gene product will accumulate in the host at high levels in a full-length and biologically active form (Baneyx, 1999). In fact, great difficulties have been encountered in achieving high yields of functional antibody fragments in defined cell locations that could simplify downstream processing. Each individual fragment demonstrates different levels of yield of soluble antibody and varying levels of toxicity to the host (Harris, 1999).

A number of limitations in *E. coli*-based expression systems and how these have been recently addressed are described below. Different strategies to obtain high levels of active antibody fragments are discussed according to the systems considerations and type of protein expression (cytoplasmic expression, periplasmic expression, extra-cellular expression, cell-free proteins).

- System considerations.

a) Plasmid loss and instability.

In order to achieve high gene dosage, heterologous cDNAs are typically cloned into plasmids that replicate in a relaxed fashion and are present at 15-60 or a few hundred copies per cell. Nevertheless, plasmid loss can increase tremendously in the case of very high copy number plasmids, when plasmid-borne genes are toxic to the host (Baneyx, 1999). The simplest way to address it is to take advantage of plasmid-encoded antibiotic-resistance and supplement the growth medium with antibiotics to kill plasmid-free cells (Baneyx, 1999).

Nevertheless, usage of antibiotic requires evidence demonstrating clearance of the antibiotic from the final product to secure regulatory approval for clinical applications. Therefore, Weir et al, 1999 patented a method for the expression of recombinant proteins such as Fab' in bacterial hosts in a defined medium in the absence of antibiotic selection. The method uses an expression vector that stably maintains a medium copy number between 6 and 50.

b) Low gene dosage.

Typically, the more rapid the intracellular product accumulation, the greater is the probability of product accumulation in inclusion bodies. However, the usual target is soluble, active antibody. The most important parameter to obtain this result is the rate of protein synthesis (translation), which depends principally on gene dosage, promoter strength, mRNA stability and the efficiency of translation initiation (Baneyx, 1999). Although many of the promoters used to drive the transcription of heterologous genes have been constructed from lac-derived regulatory elements, there is a need for promoters with little or no expression before induction and with reliable, adjustable expression (Swartz, 2001).

c) Promoters.

Lac-promoters used under control of isopropyl-B-D-1 thiogalactopyranoside (ITPG) as the inducer, can lead to large amounts of mRNA and accumulation of the desired recombinant protein of up to 50% of the total cell protein. But this can cause ribosome destruction and cell death (Baneyx, 1999). Some of the strategies developed to overcome this problem are co-over expression of a lysozyme which degrades mRNA polymerase and the insertion of a lac operator in order to reduce leaky transcription.

- Cytoplasmic expression

a) Folding in inclusion bodies and cytoplasmic degradation.

This method is relatively straightforward and hence it was the initial method attempted to produce antibody fragments.

However, antibody fragments are susceptible to proteolytic degradation by *E. coli* cytoplasmic proteases when improperly folded, as a natural mechanism of recycling useless proteins into their constituent amino acids (Baneyx, 1999). Therefore, expression of proteins is required into insoluble and inactive inclusion bodies to avoid degradation. Nevertheless, this in turn leads to the necessity of further processes for resolubilisation and renaturation of the antibody fragment.

Obtaining properly folded protein from inclusion bodies requires appropriate aggregation. The search for optimal folding conditions is still empirical with the best results obtained from evaluating a matrix of conditions affecting solubility, disulfide-bond formation and isomerization (Swartz, 2000). A traditional approach to reduce protein aggregation in the cytoplasm is through fermentation engineering by reducing the cultivation temperature by using cold-inducible promoters (Mujacic et al, 1999; Baneyx, 1999)).

b) Secreted fusion proteins.

Fusion to cytoplasmic proteins can increase concentrations of antibody fragments into inclusion bodies (Harrison et al, 1996). But soon it became apparent that certain fusion partners could greatly improve the solubility of passenger proteins that otherwise would accumulate within inclusion bodies in the cell cytoplasm. The reason could be that these fusion proteins reach a conformation that promotes the acquisition of correct structure in downstream folding units by favouring on-pathway isomerization reactions (Baneyx, 1999).

The main disadvantages of fusion proteins are: liberation of the passenger proteins requires expensive proteases, cleavage is rarely complete, additional steps may be required to obtain an active form, and solubility is never guaranteed (Baneyx, 1999).

- Periplasmic expression

a) Folding and degradation in the periplasm.

The periplasm is an oxidizing environment that contains enzymes catalyzing the formation and rearrangement of disulfide bonds (Missiakas et al, 1997). As a result, protease activity is at a minimum and a method emulating the folding and assembly pathway of antibodies in eukaryotic cells was developed (Skerra et al, 1988). Periplasmic proteins can be further translocated to the growth medium of the cell by deliberate permeabilization of the outer membrane. This process greatly simplifies the purification of target polypeptides although it increases their dilution (Baneyx, 1999).

On the other hand, over expression also leads to leakage of periplasmic enzymes to the fermentation media and after long periods of antibody production, autolysis has been reported (Somerville et al, 1994). The host strain, plasmid and growth conditions (medium composition, induction time and temperature) do appear to have an effect on the success of periplasmic expression and a wide variety of antibody fragments and antibody fusion proteins have been successfully produced (Skerra et al, 1988).

Dual Origin vectors (DUOV) have been found to be particularly suitable for expressing antibody fragments. A dual origin vector comprises two replication functions which are controlled by a temperature sensitive repressor.

Increasing the growth temperature of the medium above 34 °C in a DUOV system leads to an increase in copy number from about 5 to several hundreds per cell. This causes the repressor to be titrated out and expression of the foreign protein can take place. Weir et al, 1995 patented an invention related to a process for obtaining antibodies in soluble and correctly folded and assembled form by raising the operating temperature at some stage during the expression phase of the fermentation.

One possible cause for formation of insoluble proteins is aggregation of the heterologous polypeptide into inclusion bodies as a result of incorrect folding of the polypeptide chain.

- Extra-cellular expression

Many attempts have been made to selectively release recombinant proteins into the medium, but such systems have not been commercialized because it is difficult to control them. In one recent example, the third topological domain was secreted into the periplasm and mostly released when induced. Nevertheless, the culture suffered a three order of magnitude loss in viability (Swartz, 2000).

- Cell-free protein synthesis

Cell free methods appear to introduce a potential technology to obtain high yields of recombinant proteins but only at a high cost. In this system the cells have to be grown and cell extracts prepared. It allows the synthesis of proteins that are toxic to the cell, optimal use of metabolic resources, and flexibility to manipulate protein synthesis and folding (Nakano, 2003).

Expression of functional antibody fragments could be increased not only by varying the nature of the expression system but also by optimising fermentation parameters. Therefore, it is clear that the development of fermentation strategies is required to reach high yields of fully-functional antibody fragments in a desirable cellular location.

1.4. FERMENTATION PROCESSING OF ANTIBODY FRAGMENTS BY *E. coli*

Fermentations with inducible product expression, like Fab' antibody fragments by *E. coli*, can be divided in two main stages:

1. Production of Biomass.

In this stage it is desirable to obtain the biomass both as much and as fast as possible. Nevertheless, as already commented, an uncontrolled, fast-growing fermentation could lead to plasmid loss and as a result may cause a reduction in yield and an increase in mutation rate. Many fermentation strategies have been developed in order to limit the specific growth rate of the culture and increase the production; these strategies are generally based on using non-optimal growth conditions. For example, by reducing the temperature of the process, by using highly defined medium, or by adopting fed-batch fermentation.

Fed-batch fermentations (whereby a limiting nutrient is fed to the culture at a rate which restricts the organism's growth) are usually preferred rather than batch fermentations due to higher biomass concentrations that can be obtained. Yields of antibody fragments have been reported for batch culture ranging from 0.04 g/L (Berry et al, 1994) to 0.45 g/L (King et al, 1993).

On the other hand, fed-batch fermentations using highly defined medium can result in levels up to 20 to 40 g/L of biomass and titres up to 1 to 2 g/L (Carter et al, 1996). Using an optimised expression vector under non-limited growth conditions titres up to 3 g/L have been reported (Horn et al, 1996).

The success of fed-batch fermentations could be due to supplying a growth-limiting nutrient (usually carbon source) at an appropriate rate which avoids the production of by-products such as acetate. Acetate formation is a common problem when glucose is supplied as the carbon source in high concentrations and it has an inhibitory effect on cell growth and production (Lee, 1996). One solution is the use of alternative carbon sources such as glycerol that could reduce acetate formation (Harrison et al, 1996).

2. Induction and Production of the Antibody Fragment.

The induction process is the critical stage for the production of the antibody fragment. Many factors such as type of inducer, temperature, point of induction, and duration of the induction phase, can influence the yield of active antibody fragment.

The most commonly utilized expression systems for production of antibody fragments from *E. coli* are the *lac* and *tac* promoter system. These promoters are used for regulating the expression of recombinant proteins as they are well understood and have been extensively characterised (Donovan et al, 1996). These promoters can be induced using Isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose. IPTG is used because it is not metabolised by the cell and the level in the growth medium remains constant after induction. Nevertheless, it has a high cost which limits its use in large-scale processes. Lactose is much cheaper than IPTG, but has the disadvantage of being metabolised by the cell.

During the induction phase it is also desirable to obtain only a small increase in biomass. This is because the production of a foreign protein could place an additional burden on the host cell's metabolism and consequently might lead to plasmid loss and increased mutation rate. In order to avoid concomitant growth and antibody formation, a nutrient which is not essential to the cell and antibody fragment production, but necessary in excess during exponential growth, can be limited after the induction, e.g. phosphate or ammonia (Glick, 1995).

After successful fermentation the desired product must be separated and purified. The necessary steps to obtain these are commonly known as Downstream Processing (DSP).

1.5. DOWNSTREAM PROCESSING OF ANTIBODY FRAGMENTS

Downstream processing is usually composed of a sequence of recovery and purification operations, whose final objective is to purify the antibody fragment to a prespecified level. The aim of the recovery operation is to obtain the product in solution (Lienqueo et al, 2000). Table 1.2 summarises the different operations that can be used to recover and purify the product, depending on the final location of the antibody fragment.

The operating conditions employed in the fermentation process can affect the efficiency of the recovery stages that can also affect the purification steps. Therefore, it is important to identify the factors related not only to the yield of product, but also to the recovery and purification of it.

Table 1.2 Unit Operations for the Recovery and Purification of Fab' Antibody Fragments (adapted from Bowering, 2000; Lienqueo et al, 2000)

FRAGMENT EXPRESSION	STAGE	UNIT OPERATION	CHARACTERISTICS
Extra cellular	Recovery	Centrifugation, membrane process	Removal of cells from the medium
Intracellular (periplasm or intracellular inclusion bodies)		Homogenisation, bead milling, chemical and enzymatic lysis	Recovery of cells, cell disruption or membrane permeabilisation
		Centrifugation, membrane processes	Removal of cell debris
Inclusion Bodies		Centrifugation, membrane processes	Extensive solubilisation and renaturation:
Extra cellular/ Intracellular	Purification	Batch adsorption, ion exchange chromatography, affinity adsorption	Pre-treatments or primary isolation
		Hydrophobic interaction chromatography (HIC), high resolution ion exchange chromatography, affinity chromatography	High resolution purification
		Gel filtration (GF) HPLC, ion-exchange chromatography	Polishing of final product

1.6. METHODOLOGIES FOR THE IMPROVEMENT OF FERMENTATION AND DOWNSTREAM PROCESSES

Both the fermentation and the downstream processing must be suitable for scaling-up and scaling-down. Two key engineering aspects: mass transfer and heat transfer requirements, need to be considered for this target.

Many different methodologies have been developed in order to improve fermentation and downstream processes by finding a correlation between small and large scale and obtaining reliable and useful information for industrial processes from small scale experiments. These methodologies are known as scale-up/ scale-down techniques.

There are two main problems at industry:

a. Batch to batch variations, especially on the large scale;
b. Lack of reproducibility when the scale is changed.

Both can be addressed by scale-up/scale-down techniques which can be introduced when new processes are implemented or when established processes are being redesigned and/or optimised. The problems mentioned above involve multiple factors and presently there is no general scientific method to deal with them (Bylund et al, 2000).

Scale-up/down techniques, in which the objective is to achieve reproducibility when scaling a process, deal with the problem by selecting a criterion that should be maintained constant between scales. Unfortunately, most of the methods currently employed are based on empirical rules and therefore their success depends on the experience and knowledge of the working personnel.

On the other hand, the design of experiments methodology (DoE) offers an alternative to deal with multiple factors and their interactions. This methodology is a scientific approach to analyse the performance of experiments and consists in the appropriate planning to obtain experimental data that can be statistically analysed. Consequently, it is possible to obtain objective and valid conclusions (Montgomery, 2001).

In this work, the use of scale-up/down techniques together with design of experiments (DoE) was proposed to evaluate a Fab' fermentation process.

1.6.1. Scale-up and scale down techniques

Traditionally, in the fermentation industry, an increase of production has been usually achieved by building duplicates of existing fermenters and not by building a larger one (Oosterhuis et al, 1985). This could be due to uncertainties in scaling caused principally by transport phenomena which might lead to variations in apparent growth rates, nutrient and oxygen utilisation rates. The principal factors that are affected by scale up are summarized in table 1.3.

In order to overcome the difficulties when scaling-up, a number of scale up/down methods have been developed to predict/model the behaviour of large scale processes. Nevertheless, sometimes are failures in these methods due to inaccuracy in the models, changes in the characteristic times of different mechanisms, surface phenomena (coalescence, crystallization, wetting, foaming, etc.) or lack of knowledge of the (kinetics, fluid flow, personnel).

Table 1.3 Some factors affected by scale-up (Sweere et al, 1987)

TYPE OF FACTOR	FACTOR	CHARACTERISTICS & PROBLEMS WHEN SCALING
BIOLOGICAL	Number of generations	For production 1000 times larger than the laboratory or pilot plant, 10 additional generations are needed to reach the final stage. This increases the probability of mutation.
	Contamination probability	The increase in the number of transfer stages also increases the probability of contamination
CHEMICAL	Medium quality	For economic reasons, the use of industrial grades of raw materials is common for large scale production. This could mean variations from batch to batch
	Water Quality	The use of well or potable water is preferred rather than deionised water for production purposes. This could cause a variation in metal ions depending on the plant location.
PHYSICAL	Medium sterilisation	In large scale tanks, heating and cooling times are longer and therefore the medium quality can be affected.
	Mixing	Inadequate mixing in large scale reactors could lead to heterogeneous cultures with gradients of nutrients, pH, and dissolved oxygen, principally. Increasing the power input to the fermenter in order to improve mixing is expensive and can cause shear damage to the cells. Another important issue is the interaction between the turbulence at different scales and the morphology of the cells, especially for filamentous microorganisms.

There are many different scale-up/down methods (Sweere et al, 1987) that have been developed in order to transfer technology from laboratory scale to industrial scale, such as:

- Fundamental.

This method attempts to solve the microbalances of momentum, mass, and heat transfer. Nevertheless, a number of complications arise, such as:

a). In order to calculate the balances, this method takes into account the transport in three dimensions.

b). The balances are coupled.

c). Usually this method is set up for homogeneous ideal reactors.

- Semi-fundamental.

The solution of micro-momentum balances is avoided by using simplified flow models, generally of the following three types:

a). plug flow,

b). plug flow with dispersion,

c). well mixed

- Dimensional analysis.

It is a technique whereby dimensionless groups of parameters are kept constant during scaling. It has been used successfully although if a change of regime takes place, the method breaks down; it also can lead to technically unrealistic situations. There are many ways to generate the dimensionless groups of parameters: geometrical, fluid/solid gas properties, process variables, and dimensional constants. Nevertheless, if a relevant parameter is overlooked, an important group might be missing, resulting in an incomplete description of the process. The key issue in this method is to identify the relevant parameters usually by regime analysis.

- Rules of thumb.

The general use of a certain criterion for the same situation generates this kind of rules. All these criteria are closely related and refer mainly to oxygen transfer (pO_2 is a function of K_La which in turn is a function of Pg/V). There are many equations that are very useful for calculating the order of magnitude of different phenomena, which can be used for the design of scale-down experiments.

- Regime analysis.

The word regime refers to the dominance of a particular mechanism in the performance of a system. It is a semi-empirical approach based on identifying small-scale experiments and obtaining characteristic times to predict changes of regime on scaling. The three main questions that this method answers are:

a. Is one regime rate determining?
b. Which regime is this?
c. Does this regime change during a change of scale?

The analysis can be subdivided into:

a. Experimental methods. The basis is the variation of a characteristic parameter (e.g. velocity, concentration, particle size, temperature) to identify the influence of a certain mechanism.

b. Theoretical methods. These can be subdivided in to analytical or numerical methods, followed by a sensitivity analysis. Usually the first method can be based on time constants and the comparison of the time constants for conversion mixing, flow, diffusion, mass transfer and residence gives a good idea of which regime is rate determining.

In order to evaluate simultaneously all the factors involved in the process including their interactions, which could be more important than the factors by themselves, DoE is an appropriate methodology to utilise.

1.6.2. Design of experiments methodology (DoE)

Methods, such as DoE can greatly increase the efficiency of experiments by considering main effects and interactions between different factors on a certain response. Montgomery, 2001 proposed as a guideline the following seven points:

1. Recognition and Statement of the Problem. In this stage the question "what do I want to know when I finish my experiment?" has to be resolved. In order to do this, it has to take into account the available knowledge of the system. The knowledge of the system can have a theoretical or empirical origin, or even a mixture of both. Therefore a theoretical, empirical or semi-empirical model can be generated to simulate the process. Montgomery, 2001 recommend to have an empirical model (applicable only to an experimental region) to start with and build up later a theoretical one.

2. Selection of Factors and Levels. The variables of a system can be classified in independent or dependent. Independent variables, also named factors, are those which have a direct influence on the responses of a system. The factors and specific levels to work with during the experimentation must be selected according to the knowledge of the system, and during the first stages of the project.

3. Selection of the Response Variable. The dependant variable or response variable is the result of the execution of an experiment under certain conditions based on different factors. It must be selected according to the useful information that can be obtained from it.

4. Selection of the Model of the DoE. If the three previous steps have been done correctly, this step is relatively straightforward. The criterion used to select a model includes the size of the sample (replicates), selection of the order of experiments, and the natural experimental constraints.

5. Execution of the Experiments. It is very important to make sure that the execution of experiments is done according to the selected model; otherwise the errors could invalidate the results.

6. Analysis of Results. The statistical methods together with good engineering and knowledge of the system will allow the proposal of solid conclusions and important solutions to the problem.

7. Conclusions and Recommendations. Once the data has been analysed, the proper conclusions have to be written and the recommended future work to be proposed.

In figure 1.2 the model of a general process or system under experimentation is shown. In this model, the process or system can be shown as a combination of operational conditions, such as DOT, agitation rate, temperature, etc. to transform raw material (e.g. inoculum, media and inducer) into main product with one or more measurable responses (e.g. Fab', acetate level, glycerol utilisation, etc.).

Some of the factors of the process: x_1, x_2, ..., x_p, are controllable, while other factors: z_1, z_2, ..., z_q, are not controllable (Montgomery, 2001).

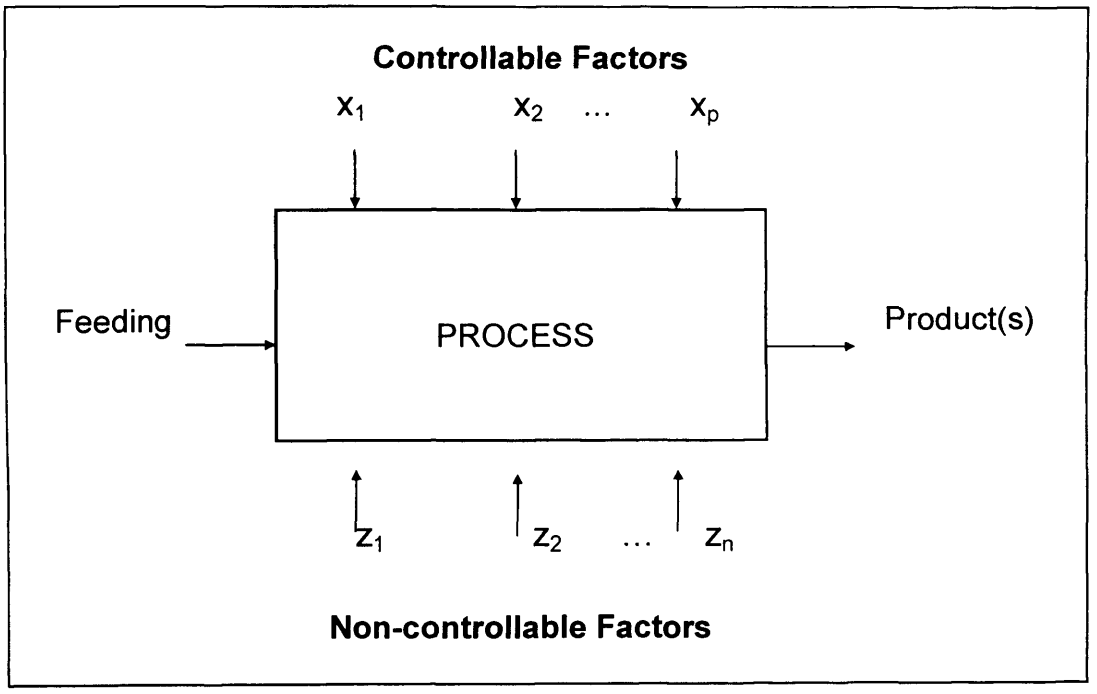


Figure 1.2 General model of a process or a system

There are many different examples of work in biological systems where DoE methodology has been used, especially in media optimisation studies. For example, the optimisation of medium components for the production of clavulanic acid by *Streptomyces clavuligerus* was performed using both fractional factorial design and response surface methodology (Wang et al, 2004). Another example is the use of a 2^2 factorial design to study the simultaneous effect of temperature and moisture on *Aspergillus niger* growth in the solid-state fermentation. The logistic model was then used to calculate growth kinetic parameters at different levels of temperature and moisture (Hamidi-Esfahania et al, 2004).

Recently, the optimisation of chitinase production using statistics based on experimental design has been reported. Preliminary studies on the factors enabled identification of eight variables important with respect to chitinase production. The most important factors with respect to each strain were then identified using the 2-level fractional factorial design (Nawani et al, 2004).

These examples indicate that DoE methodology can be very useful in organising experimental work and in the analysis of results. In the following chapter, each of the points proposed as a guideline by Montgomery (2001) is discussed for the Fab' fermentation process by *E. coli*.

Chapter 2

Design of Experiments

Experimental work in this project was carried out based on DoE methodology. As described in the previous chapter DoE methodology includes seven guideline points. The following sections describe each one of these aspects in turn.

2.1. STATEMENT OF THE PROBLEM AND THE PROJECT AIM

A number of biological and non-biological factors have been reported to be related to antibody fragments production. For example, strain selection, batch/fed-batch fermentation, temperature, time & regulation of induction (Harrison et al, 1996). On the other hand, fermentation performance at large scale has been found to be related to severe oxygen gradients (Amanullah et al, 1993) and oscillating substrate concentration (Bylund et al, 1999).

Non ideal mixing behaviour in large scale bioreactors could lead to O_2 and/or nutrient gradients that might limit fermentation performance. Methodologies used to study mixing behaviour and transfer technology from small to large scale are currently mainly empirical-based. Oosterhuis and Kossen (Oosterhuis et al, 1983) proposed a scale down technique based on regime analysis. In this method a selection of characteristic times is performed based on mass transfer and conversion processes.

The rate-determining mechanisms of the process are further studied in small scale experiments to mimic critical parameters at large scale and a new parameter set-up can then be used on the large scale based on optimisation of the small scale system. Presently, however, scaling is still largely performed without enough fundamental scientific knowledge and hence scaling techniques are very difficult to use for optimisation (Bylund, et al, 1999).

An evaluation of the effects and interactions between biological and non-biological factors on a Fab' fermentation process could lead to better understanding of the process and therefore to better control of it. The principal aim of this thesis was to evaluate the effects and interactions of O_2 and nutrient concentration oscillations on Fab' yields towards improvement of the fermentation process.

Accordingly, the following objectives were set:

1. Characterisation of a Fab' fermentation process in a non gas blending system to evaluate parameters related to O_2 limitation and nutrient concentration oscillations that could be limiting product formation

2. Characterisation of a gas blending system to evaluate the effect of oxygen transfer in the broth on product formation

3. Characterisation of a feeding strategy to evaluate effect of nutrient concentration oscillations in the broth on product formation

4. Economic analysis of the gas blending system and feeding strategy proposed to evaluate the cost implications of the process

2.2. SELECTION OF FACTORS AND LEVELS

2.2.1 Preliminary analysis

The process of Fab' antibody fragment production can be divided in two principal stages: inoculum preparation and fermentation.

The inoculum stage is subdivided into three parts (see figure 2.1):

a) Complex Medium (CM). This part is the inoculation of a vial from a working cell bank (WCB) into shake flasks with CM
b) Defined Medium (DM). This part is the inoculation of the culture in CM into shake flasks with DM
c) Fermenter. This final part is the inoculation of the culture in defined medium (DM) into the fermenter

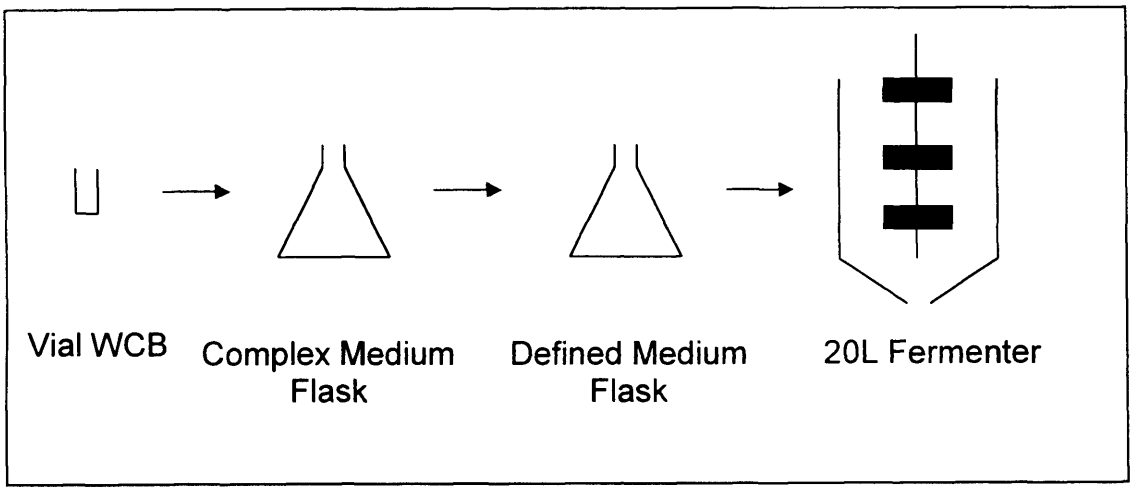


Figure 2.1 Inoculation process scheme for 20 L Fab' production

The fermentation stage is subdivided into two parts (see figure 2.2):

a) Exponential growth phase. An increase in biomass is obtained after adaptation of the inoculum. At this stage the initial C-source is consumed and further additions of C-source are required in order to obtain a high cell density before induction.

b) Induction phase. Once a certain amount of biomass is reached in the culture, the inducer is added to start the production of antibody fragments.

As can be seen in table 2.1, many different factors have been evaluated to improve recombinant protein yields in various processes. Analysis of each one of the factors considered to be evaluated in this project is discussed in the following section.

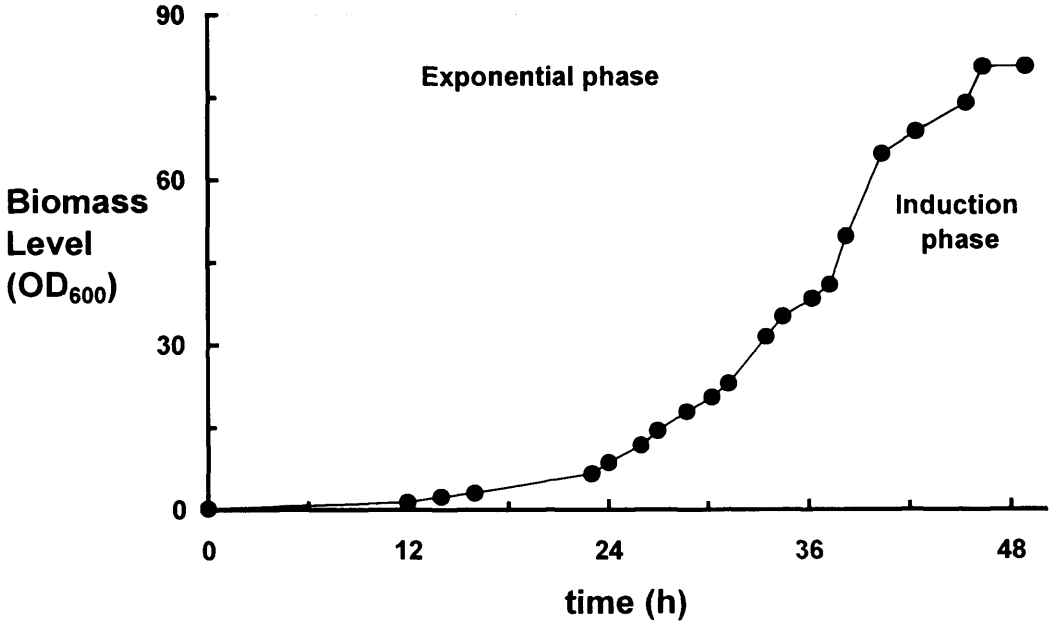


Figure 2.2 Fermentation process scheme for 20 L Fab' production

Table 2.1 Factors and levels used in fermentation processes to produce recombinant proteins by *E. coli.*

FACTOR	RECOMBINANT PROTEIN	LEVEL
Initial OD in the fermenter medium	<100 kDa	OD_{580}: 0.2-0.5 (Bylund et al, 1998)
	rhGH	10% of final total volume (7 L, 9 L, 15 L, 3000 L) (Bylund et al, 2000)
	Fab'	OD_{600}: 0.2 -0.3 (Humphreys et al, 2002; OD_{600}: 0.3 (Bowering et al, 2002)
	scFv	OD_{610}: 2 (Harrison et al, 1996)
Initial concentration of phosphates in the medium	<100 kDa	K_2HPO_4: 6g/L; KH_2PO_4: 9g/L (Bylund et al, 1998)
	rhGH	K_2HPO_4: 6g/L; KH_2PO_4: 9g/L (Bylund et al, 2000)
	Fab'	NaH_2PO_4: 2.8g/L; (Bowering, 2000) $NaH_2PO_4.H_2O$: 4.14g/L (Humphreys et al, 2002)
	scFv	$(NH_4)_2HPO_4$: 2.33g/L (Harrison et al, 1996)
Initial main C-source concentration in the fermenter medium	<100kDa	Glucose: 10 g/L (Bylund et al, 1998)
	rhGH	Glucose: 22 g/L (Bylund et al, 2000)
	Fab'	Glycerol: 30 g/L (Bowering, 2000; Humphreys et al, 2002)
	scFv	Glucose: 12.5 g/L (Harrison et al, 1996)
DOT level	<100kDa	30% L (Bylund et al, 1998)
	rhGH	not specified
	Fab'	30% (Bowering, 2000; Humphreys et al, 2002)
	scFv	20% (Harrison et al, 1996)

Table 2.1 (continuation) Factors and levels used in fermentation processes to produce recombinant proteins by *E. coli*

FACTOR	RECOMBINANT PROTEIN	LEVEL
Feeding strategy	<100kDa	Batch-fed (Bylund et al, 1998)
	rhGH	Three different stages (Bylund et al, 2000): 1) An exponential feed; 2) A constant feed when the estimated maximum O_2 transfer capacity of the reactor was approached; 3) The feed rate was decreased to 80% of its maximum value at time of induction.
	Fab'	Fed-batch (Bowering, 2000; Humphreys, et al, 2002)
	scFv	Exponential feeding adapted for fed-batch. Linear feeding was used when O_2 became limited (Harrison et al, 1996)

2.2.2 Analysis of controllable factors and levels in the inoculum stage

Three main variables were considered at this point:

1. Initial concentration of inoculum

Inoculum preparation was the first priority in order to achieve robust and reproducible fermentations. Growth kinetics studies in shake flasks were performed prior to fermentations in order to obtain an inoculum for the fermenter in the exponential phase rather than in stationary phase.

As can be seen in table 2.1, the quantity of inoculum to use in recombinant protein fermentation could be up to 10% of the medium volume. Studies in shake flasks were performed to ensure an appropriate inoculum size (see section 3.1.5).

2. Concentration of nutrients

The medium used for antibody fragment production could be highly influential on the final product yield. Both complex and defined medium have been used for batch and fed-batch fermentations, respectively. Complex medium generally support higher specific growth rates, but defined medium in fed-batch fermentations have produced the highest cell concentrations (Harrison et al, 1996).

3. Operating conditions

At this point, operating conditions such as initial pH, temperature, and agitation rate were kept constant. Many different studies have constantly reported values of pH 7, temperature of 30°C, and an agitation rate of 150-200 rpm for inoculum preparation in shake flasks (Harrison et al, 1996; Bylund et al, 1998; Bylund et al, 2000; Bowering, 2000; Humphreys, et al, 2002).

2.2.3 Analysis of controllable factors and levels in the fermentation stage

1. Concentration of C-substrate

A high concentration of the C-source can lead to fast growth of *E. coli* and production of overflow metabolites, e.g. acetate (Guardia et al, 2001).

Also, substrate gradients in large scale reactors can cause overflow metabolism. This would mean that the microorganism i) did not respond fast enough to the gradients, ii) did not respond to the rapid fluctuations with reduced growth or by-product formation, iii) the by-products could be consumed in another reactor zone or later in the process (Bylund et al, 1998).

Using glucose as the C-source has the disadvantage that it is easily metabolised and consequently the culture grows faster, which could lead to plasmid loss and acetate formation (Harrison et al, 1996). Therefore, the C-source used was glycerol to obtain a lower specific growth rate. This particular fermentation process followed a pulsed fed-batch technique (or batch-fed) with additions of glycerol based on specific OD_{600} measurements (see section 3.1.6). Two levels of initial glycerol concentration (30 and 60g/L) were studied in shake flasks and at 20 L scale when evaluating different feeding strategies (see chapter 6).

2. Concentration of nutrients

Previously, it has been reported that the concentration of phosphates might be an important factor in the production of Fab'. Bowering (2000) showed that increasing phosphate levels at large scale resulted in increased leakage of periplasmic material into the broth. In another example, biomass formation ceased and high levels of recombinant hGH were produced when adapting glucose feed and phosphate in the medium in a fed-batch fermentation (Jensen et al, 1990).

Levels of phosphate were determined throughout the fermentation in order to observe possible correlations with either biomass or product formation. Findings are discussed in detail in chapter 6 when comparing batch-fed vs. pH-stat fermentation mode.

It has been shown that addition of cations in solution before induction increase cell wall strength and consequently improve retaining the product in the periplasm (Bowering, 2000). Level of product leakage was tested throughout the fermentation in order to determine the impact of operational conditions on product loss to the broth.

3. Operating conditions

Previous work on the Fab' process in a non-gas blending system (Bowering, 2000) at UCL showed that a cascade control based on agitation rate and air flow rate was unable to maintain a constant level of DOT above zero. Therefore, it was hypothesised that problems of O_2 transfer could affect product formation.

Another important factor to evaluate, related to O_2 transfer, was agitation rate. In the non gas blending system, cascade control changed the agitation rate from 500 rpm to a maximum level of 1000 rpm in order to try to maintain a constant DOT level. In the gas blending system, a constant agitation rate was kept throughout the fermentation.

Two levels of DOT and agitation rate were evaluated at 20 L scale in a gas blending fermentation. DOT levels of 30 % and 50 %, and agitation rates of 500 rpm and 1000 rpm were studied.

Other operating parameters such as pH and temperature were kept constant in all fermentation processes. Only during a screening experiment at shake flask level was the effect of temperature on biomass levels included when evaluating possible feeding strategies by changing the initial glycerol concentration. Results are discussed in chapter 6.

2.3. SELECTION OF RESPONSE VARIABLES

The principal response to evaluate in this process was the final Fab' concentration. As discussed in the previous chapter, Fab' antibody fragments have a wide variety of applications. Therefore, further research to increase our knowledge of the fermentation process in view of the optimisation of Fab' productivity is very valuable.

Other responses such as: biomass levels, cell lysis, substrate consumption, and by-product formation, were considered and measured in order to obtain a more complete profile of the system. Analysis of different fermentation profiles were used to develop correlations between these responses and Fab' productivity.

1. Concentration of Fab' antibody fragment

Bowering, 2000 reported Fab' levels of up to 680 mg/L with 10% leakage when changing operational parameters. These changes included the switch of the main C-source from glucose to glycerol, use of phosphate limited medium, addition of calcium and magnesium prior to induction, and reduction of temperature before the induction period. However, the plasmid utilised by Bowering was different (plasmid pAGP-4), so that no direct comparison with this work could be done.

In another study, Humphreys et al, 2002 reported a yield up to 580 mg/L when optimising the balance of light chain and heavy chain expression using a different plasmid in a batch-fed fermentation without gas blending.

2. Concentration of Biomass

It was speculated that an increase in biomass concentration could lead to an increase in Fab' production. Since biomass formation could utilise metabolic resources (precursors, ATP, NAP(P)H, etc) that otherwise could be used for Fab' production. Therefore, no further growth at induction phase was intended. It was also speculated that glycerol levels had an effect on biomass accumulation in the induction phase.

3. Substrate consumption: glycerol and lactose.

Glycerol was the main C-source for the production of biomass, as lactose was mainly used during the production of Fab'. Therefore, residual glycerol in presence of lactose at the start of the induction phase could have an effect on Fab' titres and biomass accumulation. This hypothesis was tested when comparing batch-fed (with double concentration of nutrients) with pH-stat fermentations, where no residual glycerol was present at induction phase (see chapter 6).

It was also hypothesised that glycerol oscillations had an effect on the cell culture. This was tested when comparing batch-fed (normal concentration of nutrients) with pH-stat fermentations, where no glycerol oscillations were observed throughout the fermentation. Substrate profiles were determined for the complete period of fermentation at 20 L.

4. By-product formation: acetate.

An improvement in productivity could be achieved by an increase in the cell density where by-product formation has been avoided. It has been repeatedly reported that acetate not only inhibits biomass growth but also product formation in *E. coli* fermentations (Bylund et al, 1998; El-Mansi et al, 1989; Luli et al, 1990; Johnston et al, 2002). Toxic levels of acetate have been reported for different strains of ≥ 5 g/L (Lee, 1996). Acetate formation profiles where obtained for fermentations at 20 L scale.

Furthermore, the effect of a feeding strategy to minimise acetate levels due to reduction of glycerol oscillations was studied. The effect of residual glycerol in the induction phase on acetate levels and Fab' yields was also analysed when studying feeding strategies.

5. Cell Lysis

As the system studied expresses the product in the periplasmic space, cell lysis directly affects product leakage into the fermentation broth. This needs to be avoided as much as possible in order to simplify further downstream process stages.

Finally, as can be observed from the previous analysis, it is difficult to isolate the effect of one response on the other factors and on the product yield. Therefore, it is believed that for a true optimisation of this process a study of the effects and interactions of all these parameters on product formation should be done. This project mainly focused on evaluating the impact of O_2 limitation and glycerol concentration oscillations on the production of Fab'.

2.4. CHOICE OF EXPERIMENTAL DESIGN MODEL

According to the theory of DoE (Montgomery, 2001), models for a single or several factors can be applied. Most common models for a single factor are: the complete randomised design, the complete randomized design by blocks, and the simple comparison between treatments. The models used for more than one factor are: the Latin square design, factorial design, and the factorial fractional design.

A factorial design 2^2 was used in the present work. This type of model allowed the analysis of effects and interactions of two factors at two levels (high and low) assuming a linear behaviour in the range of levels selected. A linear model could be proposed from the set of experiments. This model correlates effect and/ or interactions of the controllable factors with the principal response.

A set of 4 experiments was required for each of the factorial designs proposed in order to be able to quantify effects and interactions of the factors studied on the response variables analysed. A brief description of the 2^2 factorial models utilised in each stage of this study is presented here:

a. Characterisation of a gas blending system. This stage involved studies at 20 L. According to the factors and levels chosen, the set of experiments is presented in table 2.2.

Table 2.2. Factorial 2^2 model to evaluate the effects and interactions between DOT level and agitation rate on Fab' titres at 20 L scale

Exp. #	A = impeller's speed , rpm	B = DOT, %
GB-1	-	-
GB-2	+	-
GB-3	-	+
GB-4	+	+

A= - (500rpm), + (1000rpm); B= - (30%), + (50%)

b. Characterisation of a feeding strategy. Application of a factorial 2^2 model in this stage was useful at shake flask scale to identify possible relevant variables to increase biomass levels in this fermentation process. Based on the factors and levels chosen, the set of experiments is presented in table 2.3.

Table 2.3. Factorial 2^2 model to evaluate effect and interactions between concentration of nutrients and temperature on growth kinetics in fermentations at 250 mL scale.

Fermentation	A= Glycerol Level (g/L)	B= Temperature
SF-1	-	-
SF-2	+	-
SF-3	-	+
SF-4	+	+

A= -(normal, 30g/L), +(double, 60g/L); B= -(30°C), +(37°C)

Finally, a comparison of fermentations at 20 L scale was carried out to evaluate effect feeding strategy on fermentation performance. A pH-stat feeding strategy vs. batch-fed feeding strategy was performed. Results are discussed in chapter 6.

Chapter 3

Materials & Methods

3.1. FERMENTATION PROCESS

3.1.1. Bacterial strain

E. coli strain W3110 pAC tAC 4D5 Fab' provided by Celltech Chiroscience Ltd, Slough, UK was used to prepare a working cell bank (WCB) as described in Bowering et al, 2002.

3.1.2. Preparation of master cell bank (MCB)

A vial of the *E. coli* strain was thawed at room temperature (see section 3.1.4) and 50 μL were used to inoculate 200 mL of complex medium containing 50 mg/L of chloramphenicol in a 2 L baffled shake flask. The flask was incubated using an orbital shaking incubator at 30 °C and 250 rpm

Once an OD_{600} 1.5-2.0 was reached, the necessary volume was used to inoculate 200 mL of complex medium containing 50 mg/L of chloramphenicol in a 2 L baffled shake flask to give an initial OD_{600} of 0.1 and incubated as described above.

Once an OD_{600} of 1.0-1.5 (mid-exponential growth phase) was reached, the glycerol stocks were prepared by addition of sterile 80% (w/w) glycerol to give a final concentration of 12.5 % (v/v). Aliquots of 1 mL were stored in cryovials at -70°C.

3.1.3. Preparation of working cell bank (WCB)

A vial from the MCB was thawed at room temperature and 1 mL was used to inoculate 200 mL of complex medium containing 25 mg/L of chloramphenicol in a 2 L baffled shake flask and incubated as above.

Once an OD_{600} of 1.0-1.5 (sterile complex medium used as the blank) was reached, the glycerol stocks for the WCB were prepared by addition of sterile 80 % (w/w) glycerol in water to give a final concentration of 12.5 % (v/v). Aliquots of 1mL were stored in cryovials at -70 °C.

3.1.4. Inoculum development

Cultures of 0.25 mL were first grown in 250 mL of complex medium (2xYT) in shake flasks. The starter cultures were incubated for 8 hrs in an orbital shaker (200 rpm) at 30 °C. An aliquot was taken and the optical density (OD) at 600 nm measured (expected to be OD_{600} 2.0-3.0).

Samples of 25 mL of the culture were transferred to flasks of defined medium, 250 mL working volume. The flasks were incubated for 12 hrs in an orbital shaker (200 rpm) at 30°C in order to obtain the required OD_{600} of 1.5-2.0. This provided an inoculum in mid-exponential growth phase, and an initial OD_{600} of 0.2-0.3 in the 20 L fermenter. The fermenter had an initial working volume of 12.5 L and contained the same defined medium as the shake flasks. The preparation of the medium was as described in Bowering et al, 2002.

Table 3.2 Composition of Complex Medium 2xYT

Compound	**Concentration**
Tryptone (g/L)	16
Yeast Extract (g/L)	10
NaCl (g/L)	5
Chloramphenicol (g/L)	0.025

Table 3.3 Composition of Defined Medium

Compound	**Concentration**
$(NH_4)_2SO_4$ (g/L)	5
NaH_2PO_4 (g/L)	2.8
KCl (g/L)	3.87
Citric Acid (g/L)	4
Glycerol (g/L)	30
Trace Elements* (mL/L)	10
$MgSO_4.7H_2O$ (g/L)	1
Chloramphenicol (g/L)	0.025

* see table 3.4.

Table 3.4 Composition of Trace Elements solution

Compound	**Concentration**
Citric Acid (g/L)	100
$CaCl_2.6H_2O$ (g/L)	5
$ZnSO_4.7H_2O$ (g/L)	2.46
$MnSO_4.4H_2O$ (g/L)	2
$CuSO_4.5H_2O$ (g/L)	0.5
$CoSO_4.7H_2O$ (g/L)	0.427
$FeCl_3.6H_2O$ (g/L)	9.67
H_3BO_3 (g/L)	0.03
$NaMoO_4.2H_2O$ (g/L)	0.024

The preparation of both medium is described in detail in the Batch Process Record (BPR) to produce Fab' by *E. coli* fermentation at 20L scale (see appendix B).

Based on the growth kinetics in figure 3.1, an inoculum size of OD_{600}= 2-4 could be suggested to transfer from CM to DM. Consequently, an inoculum size of OD_{600}~3 was used in all the fermentations performed in this process.

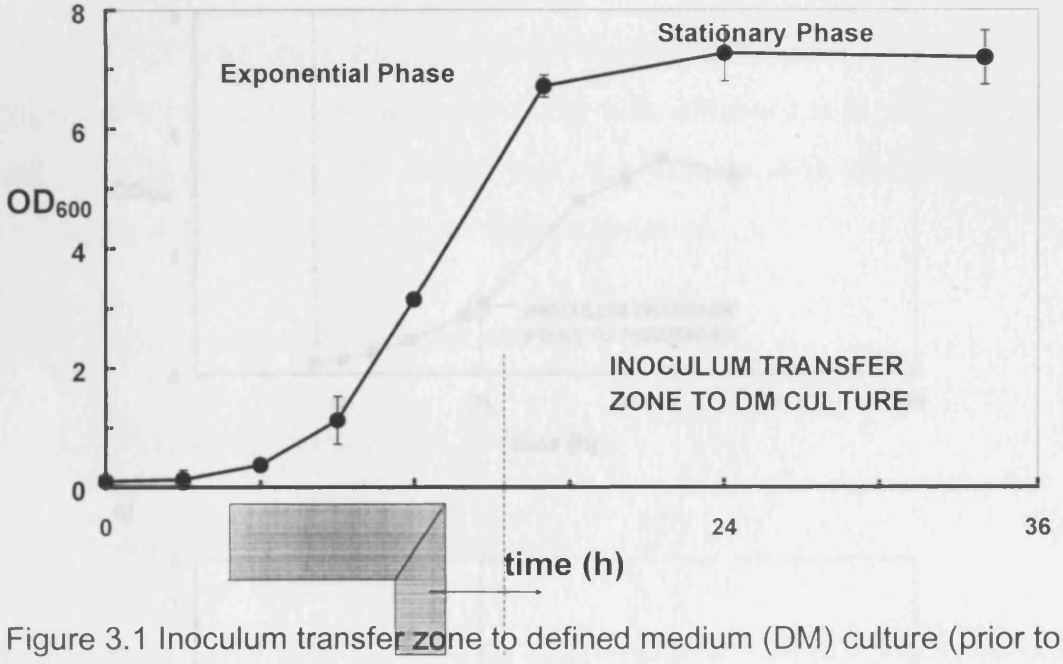


Figure 3.1 Inoculum transfer zone to defined medium (DM) culture (prior to fermentation) based on growth kinetics of *E. coli* in complex medium (CM)

In figure 3.2 the inoculum preparation growth kinetics followed in all future fermentations is presented. As can be seen, ~24 h was required to have a culture in DM at mid-exponential phase ready to inoculate the fermenter.

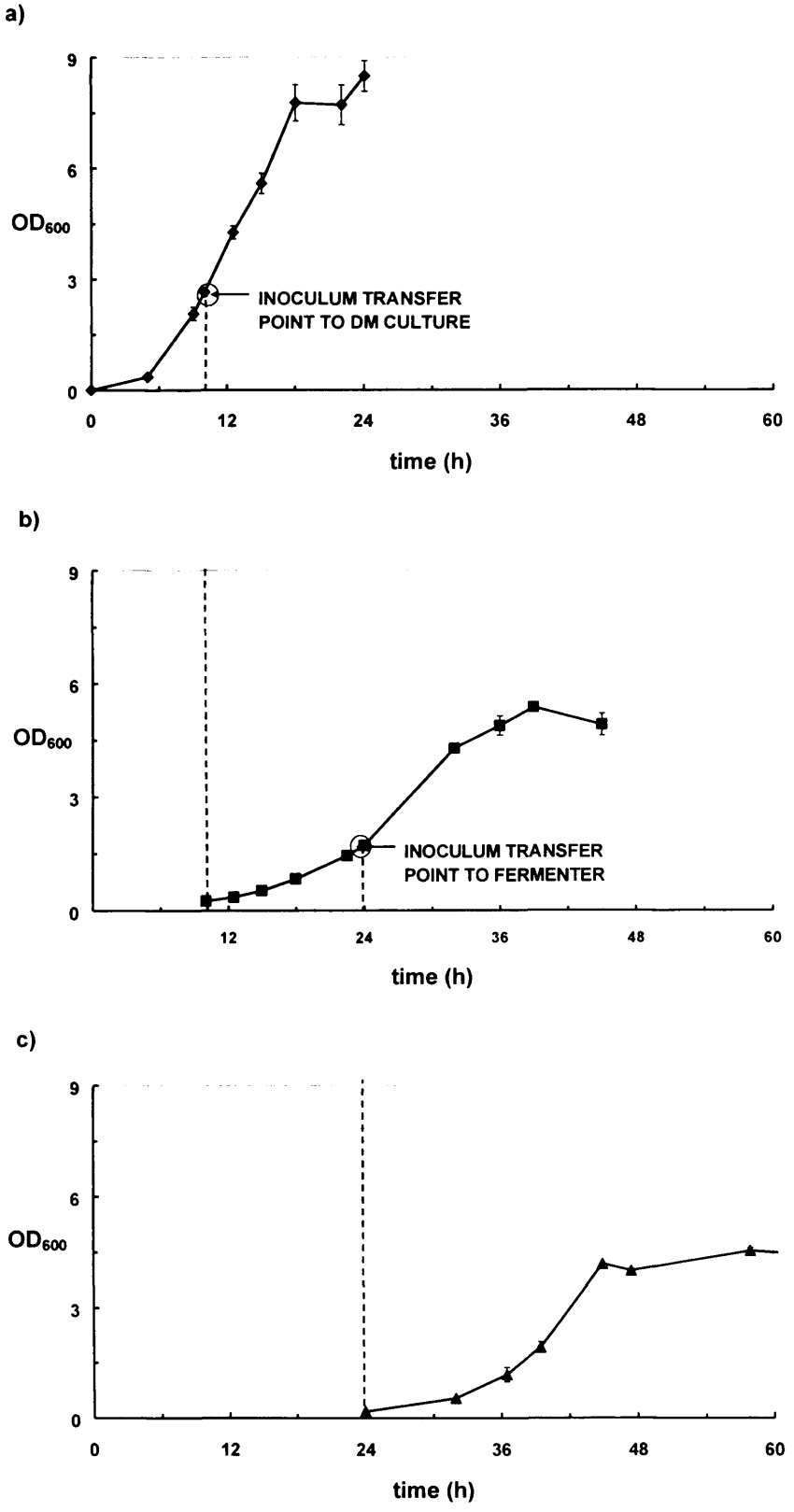


Figure 3.2 Growth kinetics of the inoculum preparation for Fab' fermentation: a) -●- CM for initial inoculum, b) -■- DM for final inoculum, c) -▲- fermenter with DM (shake flasks)

In the first stage, a fermentation time of 10 hrs was required to obtain an appropriate inoculum size to transfer into DM culture (see figure 3.2 a). In the second stage, a fermentation time of 14 h was required to obtain an appropriate inoculum size to transfer into the fermenter (see figure 3.2 b). Finally, inoculation in another DM shake flask culture was done to evaluate the possible behaviour in the fermenter. As can be observed, no lag phase was present and a μ= 0.16 +/- 0.01 was registered. This value was congruent with the ones observed in all successful fermentations at 20 L and 450 L.

Figure 3.2 was used in each fermentation to control and asses the quality of inoculum. If the inoculum size was different in a range of +/- 5 units of OD_{600} at moment of transfer, the culture was discarded. This ensured a robust and reproducible fermentation.

3.1.5. Fermenter protocol and set up

3.1.5.1. Fermentation process description

The process had three main stages:

a. 1st Stage- Transfer of Working Cell Bank (0.3 mL) from a vial into shake flasks with CM.
b. 2nd Stage- Transfer of inoculum in CM into shake flasks in DM.
c. 3rd Stage- Transfer of inoculum in DM into the fermenter

The working volumes in shake flasks and in the fermenter were 0.25 L (each stage) and 12.5 L, respectively. The volume of inoculum transferred from one stage to another was related to the amount of biomass indirectly measured by Optical Density (OD_{600}) to obtain an initial OD= 0.2-0.3 in both DM flasks and the fermenter. The transferred volume never exceeded 10% v/v.

In this Fab' fermentation, kinetics studies indicated an OD_{600} of 3.0-4.5 for CM and an OD_{600} 1.5-2.0 for DM were appropriate for obtaining a recommendable inoculum in mid-exponential growth phase after 10 to 11 hrs of incubation (see section 4.3.1).

Samples of 30 mL of fermentation broth were taken in universal bottles every four to six hours in order to measure OD_{600} and from which additions of glycerol, cations solution (magnesium sulphate and calcium chloride to enhance cell wall strength), and lactose were based for the batch-fed fermentation (see table 3.1). Assays were carried out on the samples taken to determine the concentrations of residual substrates, the levels of product formed and the biomass level achieved.

Table 3.1. Protocol of additions to the *E. coli* batch-fed fermentation at 20 L scale

Optical Density OD_{600}	**Addition made**	**Mass of glycerol, W (g)**	**Volume of water added,V (L)**	**V_{total} (L)**
15	Glycerol (30 g/L)	375	0.09	0.39
35	Glycerol (20 g/L)	250	0.06	0.26
40	Cations solution: ($MgSO_4.7H_2O$ to 14.4 mM & $CaCl_2.6H_2O$ to 1.7 mM)	44.3 5.2	0.055 0.065	0.12
50	Glycerol (10 g/L) & Lactose (50g/L)	125 625	0.03 0.625	0.13 0.94

The whole process is described in detail in the Batch Process Record (BPR) to produce Fab' by *E. coli* fermentation at 20 L scale (see appendix B).

Both types of fermentations, batch-fed and pH-stat were carried out at 20L scale in computer-controlled bioreactors (Applikon, Schiedam, Holland). The 20 L vessel was sterilised using a steam jacket and a holding time of 20 minutes at 121 °C.

The pH was measured using a pH probe (Broadley Technologies Ltd, Bedford, UK). The pH was maintained at 6.95 +/- 0.05 by computer-controlled additions of either sulphuric acid (dilution 1:20) or ammonium hydroxide (15 % v/v). The temperature was maintained at 30 °C +/- 0.5 °C by using a heated jacket. The temperature was reduced to 27 °C before induction in order to promote correct product folding. The agitation rate was set at 500 rpm. DOT was monitored using a DOT probe (Broadley Technologies Ltd, Bedford, UK). Fermenter exit/inlet gas compositions were monitored by mass spectrometry (MM8 80 Instrument, VG Gas Analysis Ltd, Middlewich, UK). Data was logged using BioXpert software (Applikon, Schiedam, Holland). The airflow rate was set at 2.5 L/min. Gas blending was achieved by mixing the inlet air with pure oxygen (BOC Gases, Surrey, UK) for the batch-fed and pH-stat fermentation alike. The valve releasing oxygen into the inlet stream was set according to a computer control so as to maintain the DOT at a constant level of 30 %. The oxygen flow rate was varied under the control of the PID controller from 0 – 2.5 L/min.

3.1.5.1 Batch-fed fermentation

Samples of 30 mL of fermentation broth were taken in universal bottles every four to six hours in order to measure OD_{600} and from which additions of glycerol, cation solution (magnesium sulphate and calcium chloride to enhance cell wall strength), and lactose were based (see table 3.1) for the batch-fed mode. Assays were carried out on the samples taken to determine the concentrations of residual substrates, the levels of product formed and the biomass level achieved.

3.1.5.2 pH-stat fermentation

For the pH-stat fermentations, a glycerol reservoir was attached to the ammonia container by coupling two pumps controlled by the computer system (see figure 3.1). Additions of glycerol were at the same rate and time as the ammonium hydroxide additions until induction with lactose at 50 g/L. At the moment of induction with lactose the glycerol reservoir was decoupled. Assays were carried out on the samples taken each four to six hours to determine the concentrations of residual substrates, the levels of product formed and the biomass level achieved.

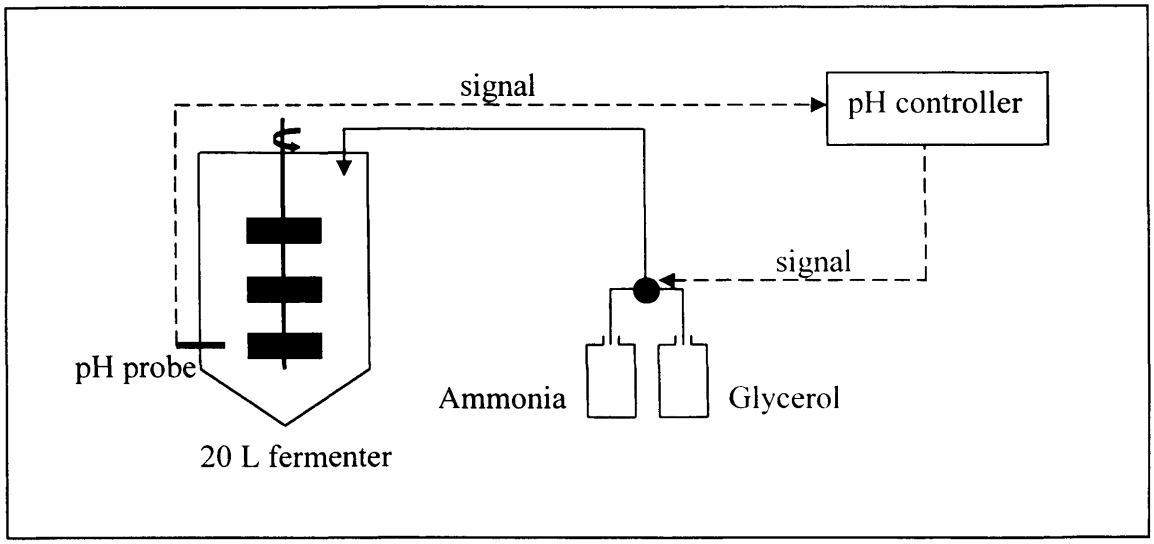


Figure 3.3. Schematic of the pH stat fermentation system for controlling glycerol additions in a Fab' fermentation process.

3.2. MONITORING OF FERMENTATION

3.2.1. Biomass measurement by optical density

The growth profile of the strain was followed by optical density measurements of the broth, measured at a wavelength of 600 nm with a spectrophotometer (DU-Spectrophotometer, Beckman Instruments Ltd., High Wycombe, UK). Samples were diluted with RO water in order that the absorbance measurement was in the linear range of the instrument i.e. 0-0.8 absorbance units.

3.2.2. Dry cell weight (DCW)

Samples of 1 mL were placed into pre-weighed Eppendorfs, and then spun down in a mini-centrifuge (Eppendorf Centrifuge 5415R, Hamburg) at 13,000 rpm for 5 minutes. The supernatant was discarded and the recovered pellets were dried to constant weight in an oven at 100 °C for 24 h. The Eppendorfs were then weighed again and the difference between the final weight and the empty Eppendorfs taken as the DCW. Assays were run at least in duplicate to ensure reproducible results within a range of +/- 5%.

3.2.3. Glycerol, lactose and acetate analysis by HPLC

Glycerol, lactose and acetate concentrations were determined via high pressure liquid chromatography (HPLC). Samples of fermentation broth, 1mL, were centrifuged at 13,000 rpm for 5 minutes, and the supernatant filtered using a 0.22 μm filter (Sigma-Aldrich, Dorset, UK).

The concentration of glycerol, lactose, and acetate in each sample was measured using refractive RI index detector (Dionex Solution GmbH, Germering, Germany) after separation with an Aminex HPX-87H column (300 mm x 7.8 mm) on a Summit HPLC system (Dionex Corp CA USA), with 5 mM sulphuric acid as the mobile phase. Glycerol and acetate concentration were determined using UV detection at 215 nm. The system was calibrated with known concentrations of glycerol, lactose and acetate each time the assay was performed. Assays were run at least in duplicate to ensure reproducible results within a range of +/- 5%.

3.2.4. Fab' concentration measurement by ELISA

Fab' concentration in the supernatant was measured by an Enzyme Linked Immuno Assay (ELISA) following periplasmic lysis as described elsewhere (Bowering et al, 2002). ELISA plates were coated (100 µL per well) overnight with HP6045 (a mouse antihuman monoclonal antibody supplied by Celltech Chiroscience, Ltd.) (2 µg/mL) in phosphate-buffered saline (PBS). After washing 4x with distilled water, serial dilutions of samples and standards were performed on the plate in 100µL sample/conjugate buffer and left to shake at 250 rpm and at room temperature for 1 h. After washing 4x with dH_2O, antibody GD12 peroxidase (The Binding Site, Birmingham, UK) was added (100 µL) and diluted 1/2000 in sample/conjugate buffer and left to shake at 250 rpm and room temperature for 1 h. After washing 4x with dH_2O, tetramethyl benzidine (TMB) substrate (10 mL acetate/citrate buffer, 100 µL 1/50 dilution of H_2O_2, 100 µL TMB) was added and the absorbance at 630 nm was recorded using an automated plate reader. The concentration of Fab' was calculated by comparison with purified Fab' standards of the appropriate isotype. Assays were run at least in duplicate to ensure reproducible results. Variation between duplicate samples was < +/- 20%.

3.2.5. Total protein assay

The Total Protein Assay was performed using a BioRad diagnostic kit (BioRad Laboratories GmbH, München, Germany) according to the manufacturer's instructions. Assays were run at least in duplicate to ensure reproducible results within a range of +/- 4%.

3.2.6. Phosphate assay

A modified malachite green method for phosphate determination which is highly sensitive and eliminates the problems of dye solubility was performed as described in Baykov et al, 1989. The method uses a colour reagent of malachite green (0.44 g) in diluted sulphuric acid dilution (60 mL in 300 mL of water) that was stable at least for one year at room temperature. On the day of use 10 mL of the dye solution was added to a solution of ammonium molybdate (2.5 mL of 7.5 % solution) and Tween 20 (0.2 mL of 11 % solution). One volume of the colour reagent was mixed with four volumes of the solution to be analysed. Absorbance at 630 nm was measured after 10 min and compared to a calibration curve. Assays were run in triplicate to ensure reproducible results within a range of +/- 5 % and a calibration curve was generated each time the assay was performed.

3.2.7 Centrifugation procedure

A CSA-1 disk-stack centrifuge (Westfalia Separator, AG, Oelde, Germany) was used during this study and was installed with a hydro-hermetic feed zone in order to minimise cell breakage during entry into the separator. The centrifuge was temperature controlled at 4°C during all experiments via cooled water circulating through the centrifuge hood.

Feed and centrate samples were retained from the initial volume of cell broth for the purpose of calculating % breakage. The centrifuge was set to operate at 9800 rpm with a corresponding corrected settling area of 1710 m^2. Material was pumped into the centrifuge at a flow rate of 60 Lh^{-1} using a peristaltic pump (Watson Marlow, Falmouth, UK, model 605DI). On reaching a full bowl (i.e. 250 g solid holding capacity) the feed pump was immediately switched off and a total bowl discharge performed. Solid material collected post discharge was re-suspended in clarified supernatant to the same initial feed concentration before conducting off-line analyses.

ELISA and the protein assay were performed on centrifuge samples to determine the level of periplasmic Fab' (product) and intracellular protein (contaminant) release associated with the continuous disk stack centrifugal recovery of cells resulting from the two feeding strategies under investigation. The centrifuge was rinsed with RO water between each experimental trial to ensure reproducible results within a range of +/- 5%. A minimum of ten bowl volumes of material were pumped through the centrifuge in between each trial in order to reach a steady-state.

3.2.8. Determination of volumetric mass transfer coefficient (K_La)

Overall volumetric mass transfer coefficients (K_La) were determined using the oxygen-balance technique as described in Bailey et al, 1986. Thus, assuming that most of the resistance to mass transfer lies on the liquid-film side, the oxygen-transfer rate per unit of reactor volume Q_{O2} is given by:

$$Q_{O2} = \text{oxygen absorption rate} = K_La\ (C^*-C_L)$$

where C^* = concentration of oxygen in the broth at equilibrium, and C_L = actual concentration of oxygen in the broth.

ESTIMATION OF OXYGEN ABSORPTION RATE

Oxygen absorption rate was assumed to be mostly due to the oxygen taken by the culture (OUR). Oxygen uptake rate was calculated from mass balances based on readings from the mass spectrometer (MM8 80 Instrument, VG Gas Analysis Ltd., Middlewich, UK) as follows:

From the mass balance of N_2

$$Gas_{IN} \%N_{2\,IN}/100 = Gas_{OUT} \%N_{2\,OUT}/100$$

$$Gas_{IN} = P/(RT) * \text{Air flow}$$

where Gas_{IN} and Gas_{OUT} are inlet and outlet gas flow per volume of reactor in $mol.L.h^{-1}$, $\%N_2$ is concentration of nitrogen in percentage volume of gases (% mol), and air flow is the set value of air per volume of reactor, P= pressure, R= constant of ideal gases, T= temperature.

Then,

$$OUR\ (mmoL.L^{-1}h^{-1}) = O_{2\,out} - O_{2\,in}$$

$$O_{2\,in} = Gas_{in}\ (\%O_{2\,in})$$

$$O_{2\,out} = Gas_{out}\ (\%O_{2\,out})$$

where $\%O_{2\,in}$ and $\%O_{2\,out}$ are the inlet and outlet concentration of oxygen in percentage volume of gases (% mol/Lh)

ESTIMATION OF THE CONCENTRATION OF OXYGEN

The driving force C^*-C_L was estimated as described by Nielsen et al, 2003. Thus, the concentrations at the gas-liquid interface can be related to each other by Henry's law as follows:

$$C^*_{O_2} = \frac{p_{O_2}}{H_{O_2}} = \frac{(C_{O_2 average})P_{tot}}{H_{O_2}}$$

where p_{O_2} = partial pressure, P_{tot} = total pressure in atm, and H_{O_2} = Henry's constant for O_2 in water at 25°C = 790.6 atmL/mol

Concentration of oxygen in the broth C_{O_2} was estimated as a fraction of the concentration of oxygen at equilibrium, as follows:

$$C_{O_2} = C^* \frac{\%DOT}{100}$$

Concentration of oxygen in the broth at 20 L scale was estimated as an average from the inlet and outlet concentration of oxygen in the air, as $C^*_{INLET} \approx C^*_{OUTLET}$. Whereas, at 450 L scale it was considered that $C^*_{IN} > C^*_{OUT}$ assuming some degree of heterogeneity in the fermenter. Therefore, oxygen concentration gradient was estimated as the logarithmic mean driving force:

$$(C^* - C_L)_{\log mean} = \frac{(C^*_{inlet} - C^*_{outlet})(1 - DOT)}{LN\left[\frac{C^*_{inlet}}{C^*_{outlet}}\right]}$$

A sample calculation for all items is presented in Appendix A.

3.2.9. Calculation of μ_{max}

The linear regression of the 1st order growth kinetics obtained from biomass measurements (OD), were used to calculate the maximum specific growth rate of *E. coli.*

$\frac{dx}{dt} = \mu x$; where x is the concentration of biomass and μ is the maximum specific growth rate (h^{-1})

Integrating,

$$\ln \frac{x}{x_o} = \mu t$$

Therefore the linearization (y = mx + b) results in,

$$\ln x - \ln x_o = \mu t$$

$$\ln x = \mu t + \ln x_o$$

3.3. SCALE-UP ANALYSIS

Scale-up analysis from 20 L to 450 L scale was performed on the basis of keeping the initial volumetric transfer coefficient constant to set up the operational parameters at 450 L scale.

ESTIMATION OF INITIAL K_La

Firstly, gassed power was estimated from the relationship developed by Michel and Miller 1962:

$$\frac{P_g}{V} = c\left(\frac{P_o^2 N D^3}{Q^{0.56}}\right)^{0.45}$$

where c = 0.72 for flat blade turbine (when the units are watt, rps, m, and m^3/s), Pg = gassed power in watt, Po = ungassed power in watt, N = agitation rate in rps, D = diameter in m, Q= air flow in m^3/s, V= working volume.

Ungassed power was estimated assuming turbulent regime (Re>10,000) from Nielsen, et al, 2003.

$$P_o = N_p \delta_L N^3 D^5$$

where N_p = Impeller power number (5.7 in turbulent regime for a single Rushton turbine according to Bailey, et al, 1986), δ_L = density of the broth in kg/m^3 (assumed similar to water), N = agitation rate in rps, and D = impeller diameter in m.

Once Pg/V was estimated, an empirical correlation for stirred vessels and coalescing (Van't Riet, 1983) was employed as a first approach to estimate the initial K_La value.

$$K_La = 0.026\ (Pg/V)^{0.4}\ (Vgs)^{0.5}$$

Where Pg/V = gassed power per volume (Watt) and Vgs= gas superficial velocity (m/s)

This correlation has been reported to be only valid for $V \leq 2600$ L; $500 < P/V < 10{,}000$ W/m^3. Values for all fermentations performed were within this range.

ESTIMATION OF CHARACTERISTIC TIMES

Circulation time (t_c) and mixing time (t_m), times were estimated based on rule of thumbs correlation as a first approach, as described by Sweere et al, 1987.

$$Nt_c = \frac{V}{2.6D^3}$$

$$t_m\ (s) = 4t_c$$

where N= agitation rate, V = working volume of reactor, D = impeller diameter.

Finally, time for oxygen consumption (t_{OC}) and time for oxygen transfer (t_{OT}) were estimated based on experimental readings from the mass spectrometer. Time for oxygen consumption was estimated from the Michaelis-Menten type equation:

$$t_{OC}(s) = \frac{K_{O_2}}{r_{O_2}^{max}} LN\left(\frac{C_{O_2,0} - C_{O_2,cr}}{r_{O_2}^{max}}\right)$$

where K_{O_2} = oxygen saturation constant, $r_{O_2}^{max}$ = maximal oxygen consumption rate, $C_{O_2,0}$ =oxygen concentration at t= 0, and $C_{O_2,cr}$ = critical oxygen concentration.

Nevertheless, assuming zero order type of kinetics if $C_{O_2} >> K_{O_2}$.

$$t_{OC}(s) = \frac{C_{O_2}}{r_{O_2}}$$

As for the time for oxygen transfer (t_{OT}), it was calculated as described by Sweere et al, 1987 as follows:

$$t_{OT}(s) = \frac{1}{K_L a}$$

where $K_L a$ is the volumetric mass transfer coefficient

A sample calculation for both characteristic times ($t_{(OC)}$ and $t_{(OT)}$) is presented in Appendix A.

3.4. STATISTICAL ANALYSIS

Statistical analysis of the results was carried out in order to quantify effect and interactions of the controllable factors chosen on the response variable selected (Fab' yield). This was done by analysis of variance (ANOVA) using the data analysis software MINITAB (Minitab Ltd., Coventry, UK).

The first step in the standard approach to analysing the experimental results from the 2^2 factorial designs was to calculate:

a. Mean $$Y = \frac{Y_1 + Y_2 + Y_3 + Y_4}{4}$$

b. Effect on response variable (Factor A) $$A = \frac{Y_3 + Y_4}{2} - \frac{Y_1 + Y_2}{2}$$

c. Effect on response variable (Factor B) $$B = \frac{Y_2 + Y_4}{2} - \frac{Y_1 + Y_3}{2}$$

d. Effect on response variable (interaction INT) $$INT = \frac{Y_1 + Y_4}{2} - \frac{Y_2 + Y_3}{2}$$

where Y_i = observations on response variable (Fab' yield) in each one of the experiments.

3.4.1 Analysis of variance (ANOVA)

ANOVA analysis was used in this work to determine effect and interactions of agitation rate and DOT level in a gas blending system. A linear model was proposed as a first approach for optimising the Fab' process

$$Y = \mu + \tau_i + e$$

where Y = response variable, μ= parameter common to all experiments, τ= parameter unique to each experiment i, e = random error.

Table 3.5 Analysis of variance for a 2^2 factorial design (Montgomery, 2001)

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_o	P_{-value}
Factor A	SS_A	a-1	$MS_A=SS_A/(a-1)$	MS_A/MS_E	<0.01
Factor B	SS_B	b-1	$MS_B=SS_B/(b-1)$	MS_B/MS_E	<0.01
Interaction	SS_{AB}	(a-1)(b-1)	$MS_{AB}=SS_{AB}/(a-1)(b-1)$	MS_{AB}/MS_E	<0.01
Error	SS_E	ab(n-1)	$MS_E=SS_E/ab(n-1)$		
Total	SS_T	abn-1			

where

$$SS_A = \frac{1}{bn}\sum_{i=1}^{a} y_{i=1}^{2}.. - \frac{y^2}{abn}$$

$$SS_B = \frac{1}{an}\sum_{i=1}^{b} y_{j}^{2}. - \frac{y^2}{abn}$$

$$SS_{Subtotals} = \frac{1}{n}\sum_{i=1}^{a}\sum_{j=1}^{b} y_{ij}^{2} - \frac{y^2}{abn}$$

$SS_{AB} = SS_{Subtotals} - SS_A - SS_B$

$SS_E = SS_T - SS_{AB} - SS_A - SS_B$

$SS_E = SS_T - SS_{Subtotals}$

a= levels of factor A, b= levels of factor B, n= experimental repetitions (e.g. duplicates)

3.5. ECONOMIC ANALYSIS

Economic analysis was done as described in García-Arrazola et al, 2001. Spreadsheets in Microsoft Excel were set up to complete the capital investment and operating costs for each fermentation strategy based on empirical factors (Peters et al, 1990). The analysis was divided into three stages:

a. Estimation of the Process Equipment Cost (PEC). This is the cost of the main equipment used in the process: a 20L scale computer-controlled bioreactor (Applikon, Schiedam, Holland).

The P1000 stirrer motor in the fermenter delivers 1000 watts of power for agitation. This information was taken into account for estimating the cost of electricity based on the amount of 2.44p/Kwh (night rate) and 6.93p/Kwh (day rate) in London in December 2003 (London Energy, London, UK). The Applikon quotation to UCL describing the fermenter and the unit price is presented in table 3.6.

Table 3.6 Bioreactor at 20L scale system cost specification

System	Unit price
20 L biobench system	£60,700.00
Packing and freight	£2, 100.00

A £5,000.00 difference was used to compare a system with no gas blending capability based on the cost of the mass flow controller and accessories (Applikon Biotechnology, Ltd., Schiedam, Netherlands).Packaging and freight and installation prices were used from the estimation by the use of factors for the installation of equipment process (see table 3.7). The steam supply was considered from the estimation for the supplements of operations (see table 3.9)

b. Estimation of the total capital investment. This value includes the economic resources invested at the beginning of the project to plan, buy, install and start running the process (fixed capital). It is also included the money needed to produce and sell the product at a constant rate (working capital). The whole estimation is based on the process equipment cost by the use of empirical factors as can be seen in table 3.7.

Four different categories of costs were estimated for the total capital investment:

Table 3.7 Estimation of the total capital to invest in a Fab' fermentation process based on empirical factors

Component	Cost (£)
EQUIPMENT OF THE PROCESS[1] National Importation	data supplied NEP IEP
a. Delivery, insurance and taxes - National equipment process - Importation equipment process	 (0.04)NEP (0.25)IEP
b. Installation of the equipment process	(0.47/1.04)EP
c. Instrumentation and control (installed)	(0.35)EP
d. Piping (installed)	(0.66/1.04)EP
e. Equipment and electrical material	(0.11/1.04)EP
f. Services facilities (installed)	(0.7/1.04)/EP
g. Buildings	(0.18/1.04)EP
h. Yard preparations	(0.1/1.04)EP
i. Land	(0.06/1.04)EP
DIRECT TOTAL COST OF THE PLANT	A= a+b+c+d+e+f+g+h+i
j. Engineering and supervision	(0.96)A
k. Costs of construction	(0.119)A
INDIRECT TOTAL COST OF THE PLANT	B= j+k
TOTAL COST OF THE PLANT	C= A+B
l. Honoraries	(0.05)C
m. Unexpected	(0.1)C
n. Other costs[2]	(0.15)C
TOTAL COSTS THAT ARE NOT OF TO THE PLANT	D= l+m+n
FIXED CAPITAL INVESTMENT	E= C+D
WORKING CAPITAL INVESTMENT[3]	F
TOTAL CAPITAL INVESTMENT	G= E+F

[1] without delivery from the suppliers

[2] previous costs and starting of the plant were considered 5 and 10% of the total cost of the plant, respectively

[3] considering starting operation conditions of 60 % of design capacity

a. Direct costs of the process, including the following elements:

- Transport, insurance and taxes of the process equipment. This cost involves the transportation cost from suppliers to the installation place and includes import taxes and insurance.
- Installation of the process equipment. This cost involves the whole installation of the equipment.
- Instrumentation and control. This cost considers the acquisition, installation and calibration of controls, including hardware.
- Piping. Considers the pipe lines required.
- Electrical equipment. This cost consists in motors, electrical material and labour required.
- Services. It includes services such as refrigeration and fuel, etc.
- Building (including services). It includes process building, auxiliaries, maintenance workshop, etc.
- Preparation of land. The preparation of the land involves cleaning, etc.
- Land. It includes licences, and cost of land.

b. Indirect costs of installation, with the following elements:

- Engineering and Supervision. This cost considers administration and engineering of supervision and inspection.
- Construction expenses. This includes operation, construction and maintenance.

3. Costs that are not from the bioprocess, according to the following concepts:

- Honoraries. This cost includes the payment at different stages of the project.
- Unexpected. Usually it is included to compensate unpredictable events, Such as strikes or natural disasters.
- Other expenses. This includes investments due to patents, research and development, etc.

4. Working capital. This includes inventories of raw material, production in the process and product finished bills to pay, and "cash in hand".

c. Estimation of the process production cost. This final value is the cost of the process over a period of time. As can be seen in table 3.8, the calculation is based on direct production costs: expenses related to materials and services, operating labour and supervisors. It also includes indirect production costs: fixed expenses such as depreciation and amortization, rent, land taxes and insurance (independent from production).

The information to start the cost estimation included the following items:

1. Cost of Raw Materials. This is the cost of the defined medium used per fermentation.

2. Cost of catalysts and additives. Antibiotic, polypropylene glycol (PPG), and the glycerol and lactose additions needed throughout the process were considered here.

3. Cost of services. This mainly represents the cost of electricity to operate the fermenter. Other services such as steam supply, heating and cooling were estimated based on factors (see services facilities in table 3.7).

Total cost of these four elements per fermentation run was based on table 3.8, where unit cost and concentration of material used in each fermentation is summarised.

Other costs were also estimated based on empirical factors to calculate the fermentation product cost:

1. Royalties. This was not considered in this case, assuming that the bacteria strain has been developed by the same holders of the process.

2. Direct cost of operators. This represents the necessary labour to operate the fermenter.

3. Rent. This was not taken into account as the acquisition of the land has been already considered in the investment of total capital.

4. Other indirect expenses. This includes expenses not essential for productions. These expenses were not considered.

5. Costs due to inventories. This represents the initial and final inventories of production of process in the process and finished product. None was considered in the estimation.

6. Annual inflation. This is considered mainly for a financial evaluation. Based on the aim of the exercise and information available, this concept was not considered.

Table 3.8 Unit cost of materials used in Fab' fermentations at 20 L scale

Concentration	Material	Unit cost, £ (amount)
	COMPLEX MEDIA	
16 g/L	Tryptone	32.9 (500 g)
10 g/L	Yeast extract	45.9 (500 g)
5 g/L	NaCl	14 (1 kg)
	DEFINED MEDIA	
5 g/L	$(NH_4)_2SO_4$	27.2 (1 kg)
2.80 g/L	NaH_2PO_4	15.7 (500 g)
3.87 g/L	KCl	28.37 (1 kg)
4 g/L	Citric acid	14.86 (500 g)
30 g/L	Glycerol	52.73 (2.5 L)
10 mL/L	Trace elements solution	
	Citric Acid (100 g/L)	14.86 (500 g)
	$CaCl_2.6H_2O$ (5 g/L)	16.6 (500 g)
	$ZnSO_4.7H_2O$ (2.46 g/L)	20 (500 g)
	$MnSO_4.4H_2O$ (2 g/L)	32.55 (500 g)
	$CuSO_4.5H_2O$ (0.5 g/L)	7.1 (250 g)
	$CoSO_4.7H_2O$ (0.43 g/L)	11.7 (100 g)
	$FeCl_3.6H_2O$ (9.67 g/L)	14.4 (500 g)
	H_3BO_3 (0.03 g/L)	13 (500 g)
	$NaMoO_4.2H_2O$ (0.02 g/L)	30.4 (100 g)
	ADDITIVES	
0.025 g/L	Chloramphenicol	6.89 (5 g)
1 g/L	MgSO4.7H2O	13.71 (500 g)
2 mL/ run	PPG	43 (1 L)
~700 mL/run	Acid	11.4 (2.5 L)
~750 mL/run	Base	70.3 (6 L)
700 g/ run (batch-fed) 600 g/ run (pH-stat)	Glycerol supply (δ=1.262 g/L)	52.73 (2.5 L)
	CATIONS SOLUTION	
	$CaCl_2.6H_2O$ (0.4 g/L)	16.6 (500 g)
	$MgSO_4.7H_2O$ (3.5 g/L)	13.71 (500 g)

Table 3.8 (continuation) Unit cost of materials used in Fab' fermentations at 20 L scale

Concentration	Material	Unit cost, £(ammount)
~50 g/L (batch-fed) ~100 g/L (pH-stat)	Lactose	35.6 (5 kg)
½ tank/run (batch-fed) 1 tank/run (pH-stat)	O_2 supply	3.93 (1 tank)
48 h/run (fed-batch) 60 h/run (pH-stat)	Electricity[1]	0.0693 (1 Kwh $_{day\text{-}time}$)

[1] A night-time rate of 2.44p exists: nevertheless, day-time rate was only used to simplify calculations and estimate maximum cost for services.

After processing the previous data, direct and indirect operating costs are estimated as follows (see table 3.9):

1. Direct operating costs.

a. Direct materials and services.

- Raw materials. Data supplied.
- Catalysts and additives. Data supplied.
- Energetic and services. Data supplied.
- Maintenance. Expenses needed to keep the operation of the process efficient.
- Supplements of operation. These are the materials required to keep the process efficient, such as: spare parts, lubricant, steam, etc.
- Laboratory expenses. This includes the expenses to control and test the quality of the product.
- Royalties. Data supplied.

b. Direct labour, which includes:

- Operation. Data supplied.
- Supervision of operation.

2. Indirect cost of operation.

Fixed expenses

- Depreciation and amortization. These are accounting terms used to distribute, during the useful period of the project, the recuperation of the investment realised.
- Rent. Data supplied.
- Land taxes. The magnitude of taxes depends on the location of the property and local law, therefore it was not considered for this study.
- Insurance. Insurance rates normally depend on the kind of process and kind of security systems installed. Nevertheless, it was considered as a percentage of the fixed capital investment.
- General expenses of the process. These are not directly related to the production process but are indispensable to keep the process at operating conditions; e.g.: medical services, security, etc.

3. Initial and final inventories of operation. Data supplied.

4. Operational cost. This is the result of considering the direct and indirect costs of production after the initial and final inventories of the process production.

5. General expenses. Such as:

- Administrative expenses. This includes salaries of secretaries, accountants, etc., but was not considered in this exercise.
- Distribution and sales. These were not considered for this case.
- Research and Development studies. This is an important support of develop new methods of operation.

6. Total cost of process. This includes costs of operation, general expenses and a percentage of unexpected expenses.

As can be observed, the main equipment cost (20 L fermenter), raw material (media components), additives (glycerol, acid and base, mainly) and services (electricity costs) were used to estimate fixed capital and cost of operations based on empirical factors (Peters et al, 1990). A final value of process cost in £/ mg of product was estimated. The effects of gas blending and feeding strategy at 20 L scale on the final product cost were evaluated when comparing: batch-fed non gas blending, batch-fed gas blending, and pH-stat gas blending fermentations.

Table 3.9 Estimation of a Fab' fermentation production cost based on empirical factors

Component	Cost (£)
I. DIRECT MATERIALS AND SERVICES	I= a+b+c+d+e+f+g+h+i+j
a. Raw materials[1]	data supplied
b. Additives[1]	data supplied
c. Inducer (lactose)[1]	data supplied
d. Oxygen[1]	data supplied
e. Electricity[1]	data supplied
f. Services[1]	data supplied
g. Maintenance	(0.06)E (from table 3.6)
h. Supplement of operation	(0.15)g
i. Laboratory expenses	(0.15)k
j. Royalties	not considered
II. DIRECT LABOUR	II= k+l
k. Operation[3]	data supplied
l. Supervision	(0.15)k
DIRECT COST OF OPERATION	I + II
III. FIXED COST	III= j+k+l+m
m. Depreciation and repayment	see note [2]
n. Rent	not considered
o. Taxes	(0.02)E (from table 3.6)
p. Insurance	(0.01)E (from table 3.6)
IV. GENERAL COSTS OF PROCESS	(0.6)(g+k+l)
V.OTHER INDIRECT COSTS	not considered
INDIRECT COSTS OF OPERATION	III+IV+V
Initial and final inventory of operation	not considered

[1] assuming 55 runs per year for a batch-fed and pH-stat fermentation (considering 2-3 days of fermentation process and 2-3 days of DSP in both strategies)

[2] assuming 20% of total direct cost of the plant, except buildings, yards preparation and land (5% was considered), 10 % of total indirect costs, and 10 % of costs that are not of the plant

[3] assuming a salary of £10/h and only one operator

Table 3.9 (continuation) Estimation of a Fab' fermentation production cost based on empirical factors

Component	Cost (£)
OPERATING COSTS	I+II+III+IV+V
VI. Operation expenses	not considered
VII. Research and Development costs	(0.5)(g+k+l)
VIII. Unexpected	see note[4]
GENERAL EXPENSES	VI+VII
TOTAL PRODUCTION COST	TPC= I+II+III+IV+V+VI+VII
Fab' PRODUCTION COST IN £/L	TPC/ working volume
Fab' PROCESS YIELD IN mg/L[1]	FPY= data supplied
Fab' PRODUCTION COST IN £/mg	TPC/FPY

[4] assuming 3 % of the process cost and general costs

Chapter 4

Characterisation of fermentation process in a non-Gas blending system

4.1. ABSTRACT

Batch-feeding fermentations at 20 L and 450 L scale were carried out to provide a base-line and a starting point to compare laboratory scale with pilot-plant scale. Results showed that cascade control was not sufficient to maintain a constant DOT level throughout the fermentation. DOT levels dropped to zero during the induction phase and during the exponential growth phase in the 20 L and 450 L fermentation, respectively. Regime analysis was performed based on experimental K_La determination. K_La values of ~400 h^{-1} were observed at 20 L and 450 L scale. Comparison of time of oxygen consumption and time of oxygen transfer suggested that O_2 limitation is present in this fermentation process and worsen as the scale increases. Mixing time was estimated from empirical correlations and values of 3 and 8 s were found at 20 L and 450 L, respectively. Comparison of mixing time with time of oxygen consumption and oxygen transfer suggested no problem due to mixing conditions until late in the fermentation at 20 L scale. Nevertheless, problems due to mixing conditions were found to be likely to occur at 450 L scale.

4.2. INTRODUCTION

Many large scale processes give a lower yield than is expected from laboratory scale experiments (Sweere et al, 1987). This is mainly due to changes of hydrodynamic characteristics related with mixing.

Small scale fermenters can be used with high power input, resulting in rapid mixing and high mass transfer. Large scale fermenters present power input restrictions for economical and mechanical reasons, leading to mixing and mass transfer problems.

The accumulation of biomass leads to continuous modification of the medium rheological properties, producing the appearance of the heterogeneous regions in the bioreactor (Ca°Caval et al, 2001). Therefore, many attempts have been made over the last 50 years to develop scale-up procedures which give an adequate estimation of the performance of production-scale fermenters based on small-scale investigations (see section 1.6).

Mixing is fundamental in biotechnology, since it defines the environment that the cells experience during fermentation. Poor mixing conditions can lead to oxygen and nutrient gradients that could be detrimental for the process performance. Therefore, mixing time has been introduced as a measure of the rate of mixing and can be considered as the time needed to obtain a certain degree of homogeneity (Sweere et al, 1987). Nevertheless, this characteristic time has the intrinsic limitation that mixing is characterised on the scale of the fermenter, and not locally (Guillard, 2003).

In 1985, Oosterhuis et al. introduced a procedure to scale-up and optimise reactors by theoretical regime analysis. Comparison of mixing time and oxygen transfer time was done in the gluconic acid fermentation by *Gluconobacter oxidans* in order to suggest that insufficient oxygen supply due to poor mixing was the rate limiting mechanism.

Mixing time is a useful quantity for measurement of mixing in the blending phase and in industrial fermentations it can easily exceed 100 seconds (Schilling et al, 1999). However, it is difficult to compare the values obtained by different researchers due to strong dependence on the definition and method of measurement of the system non-homogeneities (Manna et al, 1997).

Two approaches have been reported for scaling up processes using regime analysis and mixing times. The first one is by evaluating oscillations due to mixing problems, especially at feeding zones. The second one is by comparison of ideal and non-ideal mixing conditions in fermenters. For example, experiments using mixing time as the parameter to control have been performed in order to identify and describe the oscillating environment in *E. coli* fermentation, finding times of about 10 s (Bylund et al, 1999). Another example is the comparison of two designed model reactors at 42 L scale with two different mixing times, 10 s vs. 130 s. Results showed that this scale-down approach was successful to mimic mixing problems that lead to lower L-lysine formation in a *Corynebacterium glutamicum* fed-batch fermentation (Schilling et al, 1999).

This study presents a scale up analysis for a Fab' fermentation process based on K_La values and regime analysis. Comparison of mixing time (t_m) with time for oxygen consumption in the broth (t_{OC}) and time for oxygen transfer from the broth to the cells (t_{OT}) was performed to identify process parameters conditions that might be limiting product formation. Biomass formation, dissolved oxygen tension (DOT), air flow rate, agitation rate, oxygen uptake rate (OUR) were evaluated to complete the characterisation of this Fab' fermentation at 20 L and 450 L scale in order to propose strategies to improve the performance of the process.

4.3. RESULTS AND DISCUSSION

4.3.1. Fermentation development

Batch-feeding or pulsed fed-batch fermentations to produce Fab' at 20 L scale were carried out according to the Batch Process Record (see appendix A) at 20 L and 450 L scale. Characterisation of both systems was performed based on off-line and on-line profiles.

4.3.1.1. CHARACTERISATION OF FERMENTATION AT 20 L SCALE

As can be observed in figure 4.1 a, the first 14 h showed no significant increase of biomass levels and consequently low levels of OUR and CER were observed. The fermentation started to reach maximum growth rate of ~0.17 h^{-1} after 14 h. As a result of the decrease in DOT level, an increase of the stirrer speed using cascade control to keep constant level of 30% started. The cells reached a specific OD_{600}= 15 and the first addition of glycerol was made (see figure 4.4b). Further additions of glycerol were done in order to reach a high cell density. Glycerol profiles were obtained and are presented when discussing feeding strategies in chapter 5. The maximum cell density was ~30 g/L (DCW).

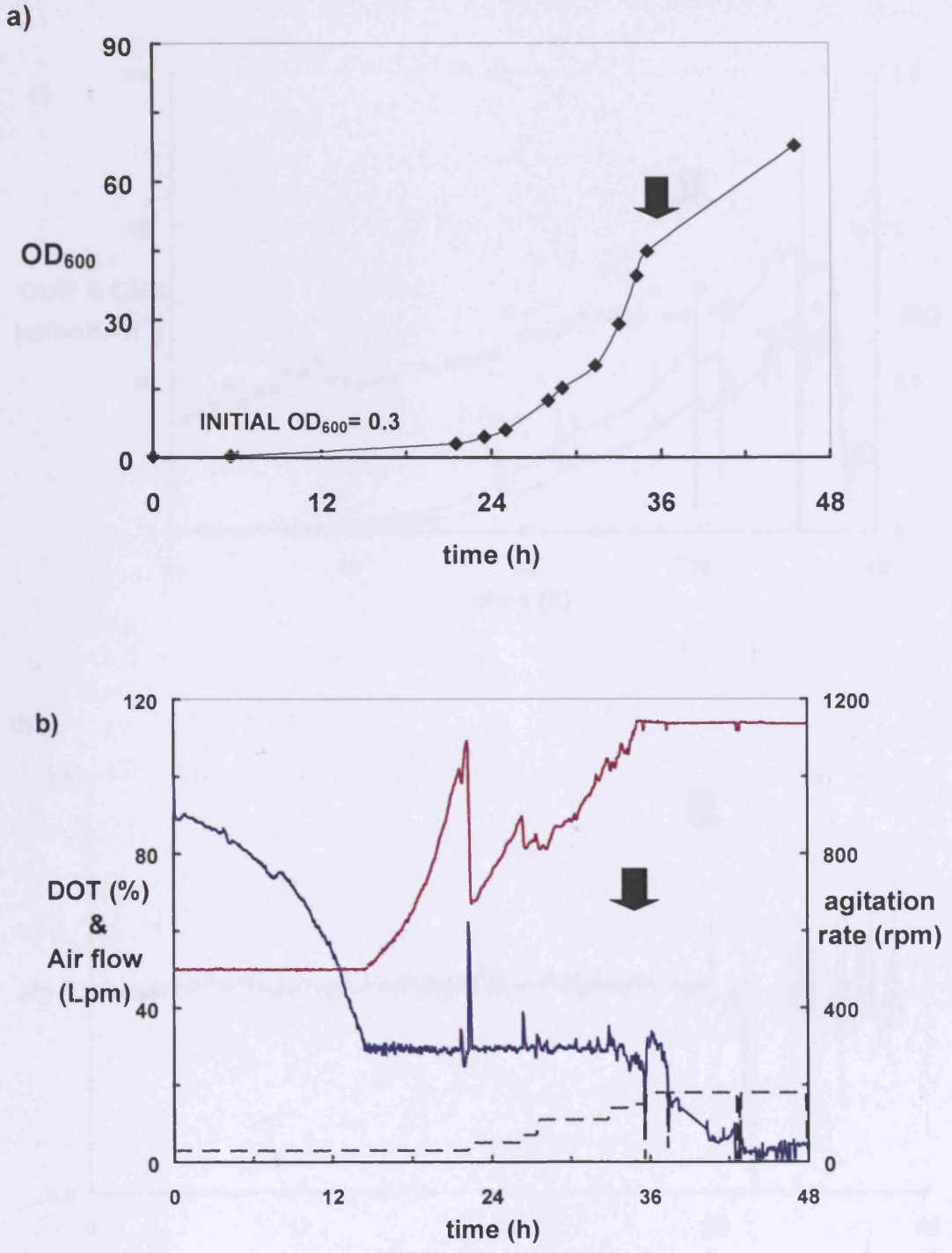


Figure 4.1 a) off-line data of ◆ biomass levels, and b) on-line data for — dissolved oxygen tension (DOT), —.agitation rate, and ---- air flow rate in a batch-feeding fermentation process to produce Fab' at 20 L. Arrow indicate addition of lactose.

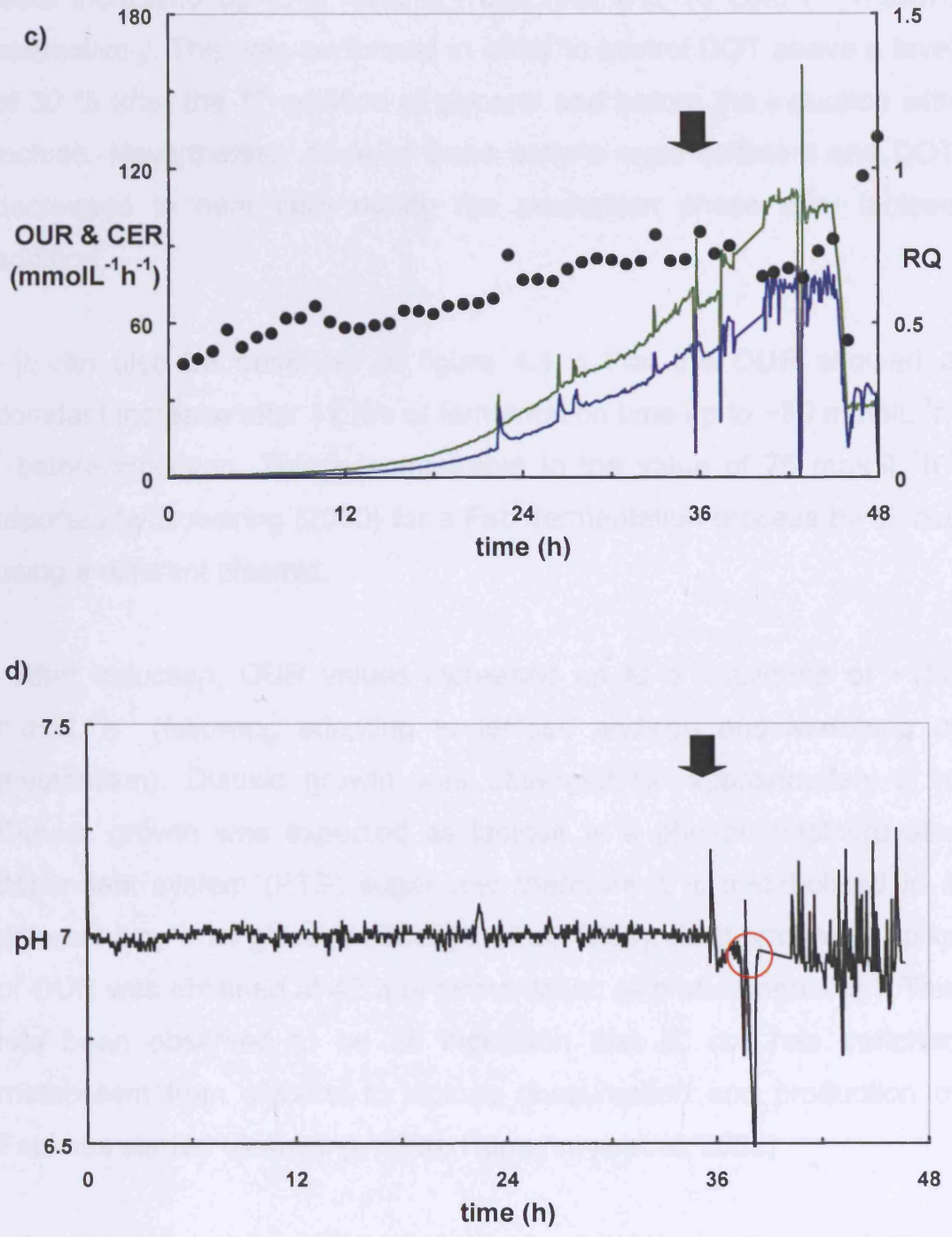


Figure 4.1 c) On-line data of — OUR, — CER, • RQ and d) On-line data for — pH level, in a batch-feeding fermentation process to produce Fab' at 20 L scale. Arrow indicates addition of lactose.

As can be seen in figure 4.1 b, both stirrer speed and air flow rate were increased up to a level of 1000 rpm and 15 Lpm (~ 1 vvm), respectively. This was performed in order to control DOT above a level of 30 % after the 1st addition of glycerol and before the induction with lactose. Nevertheless, none of these actions were sufficient and DOT decreased to near zero during the production phase after lactose addition.

It can also be observed in figure 4.1 c that the OUR showed a constant increase after 12 hrs of fermentation time up to ~80 $mmolL^{-1}h^{-1}$ before induction. This is comparable to the value of 75 $mmolL^{-1}h^{-1}$ reported by Bowering (2000) for a Fab' fermentation process by *E. coli* using a different plasmid.

After induction, OUR values increased up to a maximum of ~100 $mmolL^{-1}h^{-1}$ (following adapting to lactose addition and switching of metabolism). Diauxic growth was observed for approximately 2 h. Diauxic growth was expected as lactose is a phosphoenolpyruvate-dependant system (PTS) sugar and therefore it is metabolised in a different way than glycerol (Straight et al, 1989). Furthermore, a spike of OUR was obtained at 42 h of fermentation (4 h after induction). This has been observed to be an indication that *E. coli* has switched metabolism from glycerol to lactose consumption and production of Fab' has started (Bowering, 2000; Humphreys et al, 2002).

A pH peak was also observed at 38 h of fermentation (see figure 4.1 d). This pH increase is likely due to consumption of amino acids for energy generation and the production of ammonia ions associated with proton symport for lactose uptake (Straight et al, 1989). As cells adapted to lactose metabolism, medium pH again dropped. Nevertheless, unstable pH behaviour was observed after induction.

The unstable pH could be an indication that the switch of metabolism was not completely established in the fermentation possibly due to inhibitory effects as a result of inadequate O_2 supply, particularly after induction where oxygen demands are greatest, just prior to the carbon source switch and the start of product formation.

An RQ value of 0.7 was observed during the growth phase of all fermentation processes. This is congruent with values reported by Bowering (2000). Nevertheless, following glycerol depletion and the onset of lactose metabolism the RQ was expected to increase to 1.0. This did not happen in this fermentation and could be another indication that stable switch of metabolism was not achieved.

A total yield of 35mg/L of Fab' at 20 L fermentations in a non-gas blending system was found. This is a very low level compared to the 600 mg/L reported by Bowering (2000) using the same strain with a different plasmid. Nevertheless, results were reproducible and therefore it was hypothesised that product formation might be a consequence of insufficient oxygen supply during the induction phase due to insufficient DOT control. Further studies in a gas blending system were carried out to test the hypothesis that O_2 supply has an effect Fab' yield. Results are presented in chapter 5.

4.3.1.2. CHARACTERISATION OF FERMENTATION AT 450 L SCALE

A Fab' fermentation at 450 L scale was carried out to evaluate the effect of scale on growth and product kinetics and to produce enough material for the IMRC program at UCL. Scale-up from 20 L to 450 L was based on similar K_La values at the beginning of the process. Values of ~400 h^{-1} were observed during growth phase at both scales, indicating an appropriate scale-up of the fermentation process. Results are presented in section 4.3.2.

As can be seen in figure 4.2 a, the stationary phase was reached after ~30 h. By comparison, biomass levels (of ~20 g/L) at 450 L scale were ~30 % lower than at 20 L scale. This could be explained by the fact that culture at 450 L scale was exposed to longer periods of lack of O_2 than at 20 L scale. Insufficient oxygen supply has been previously reported by several authors as having an inhibitory effect on biomass levels (Luli et al, 1990; Johnston et al, 2002)

Cell lysis was observed near the end of the process, possibly due to O_2 limitation in the 10 h after the first addition of glycerol. Agitation rate and air flow rate were increased to a level of 500 rpm and 500 Lpm (~1.4 vvm). Nevertheless, DOT levels could not be kept constant and a drop to zero was unavoidable before the second addition of glycerol (see figure 4.2 b). This was similar to the results found at 20 L scale.

A maximum OUR value of 100 $mmolL^{-1}h^{-1}$ was obtained during the fermentation (see figure 4.2 c). This value was similar to the one observed at 20 L scale and is congruent with the fact that on both scales a maximum specific growth rate of $\mu = 0.17 \pm 0.01\ h^{-1}$ was determined.

Values of RQ showed similar behaviour to the ones at 20 L scale. A constant value of 0.7 was obtained during the growth phase. But in contrast to the 20 L scale fermentation, an increase in RQ to 1.0 was observed late after induction and towards the end of the fermentation process. This might be an indication of switch of metabolism from glycerol to lactose as the main carbon source. As can be observed in figure 4.2 d, the switch of carbon source did not take effect until late in the fermentation (~40 h i.e. after 10 h of first lactose addition). The late switch of pH could be the result of lesser metabolic activity due to cell lysis observed by decreasing values of OUR and CER.

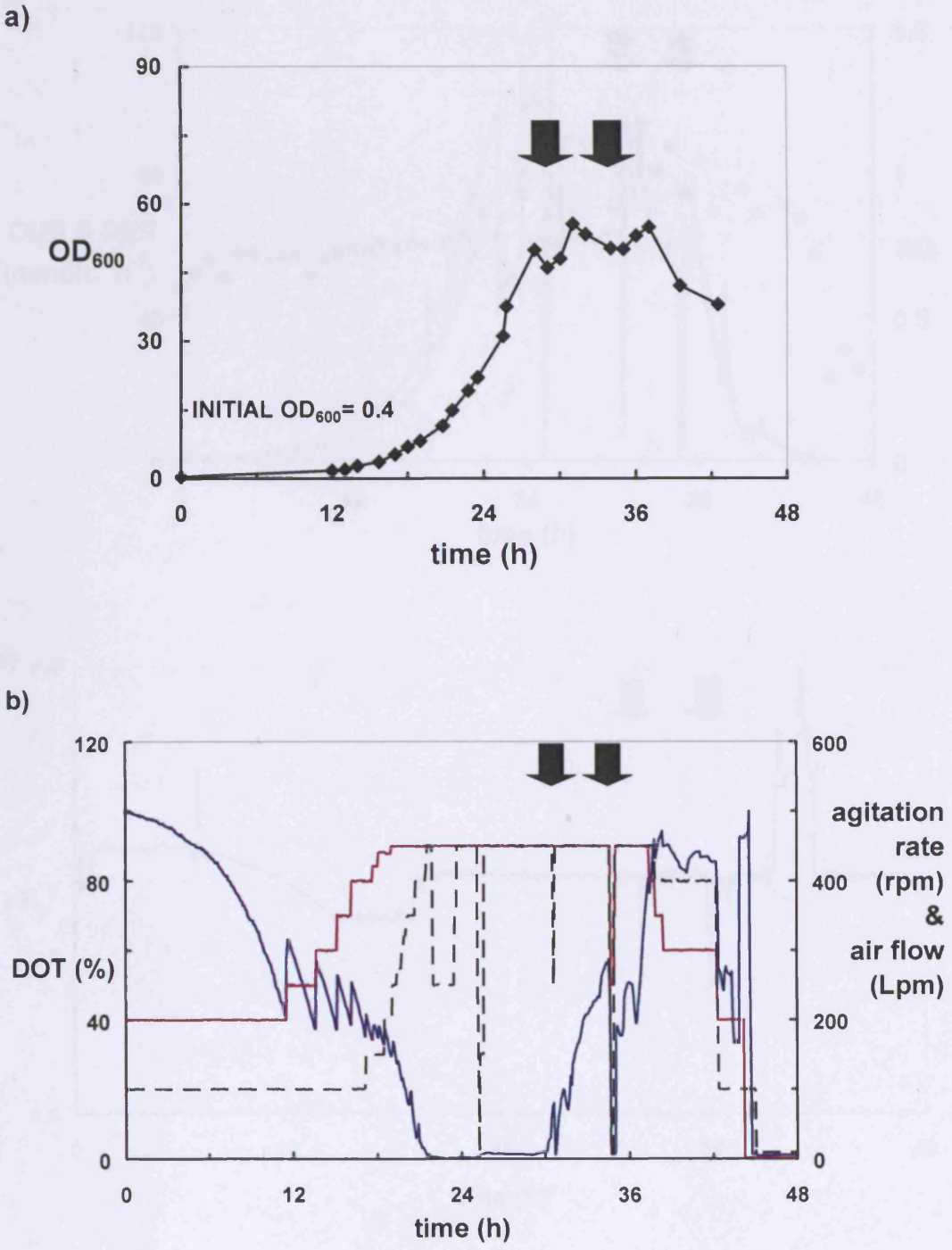


Figure 4.2 a) off-line data of ◆ biomass levels, and b) on-line data for — dissolved oxygen tension (DOT), —.agitation rate, and ---- air flow rate, in a batch-feeding fermentation process to produce Fab' at 450 L scale. Arrows indicate additions of lactose.

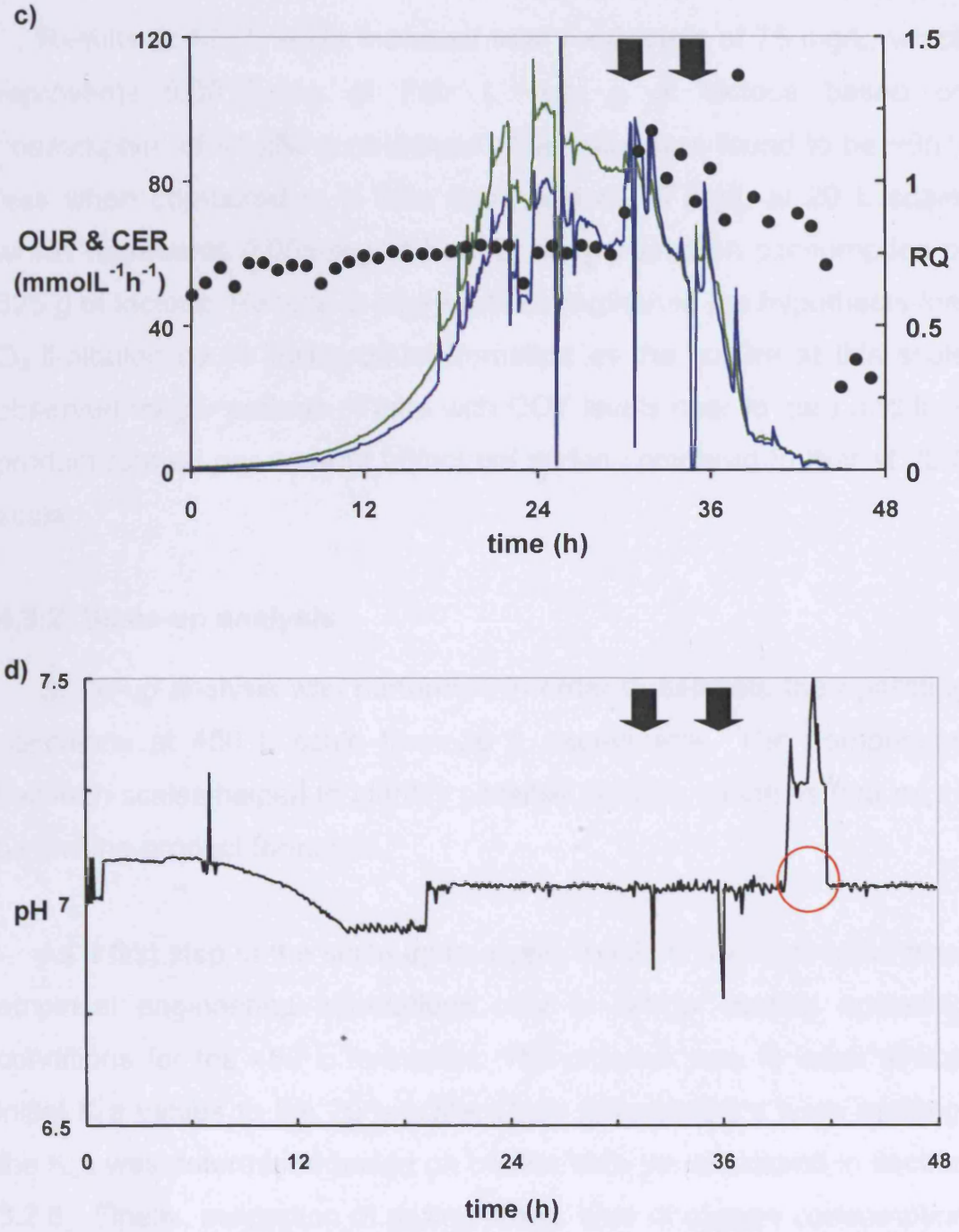


Figure 4.2 c) On-line data of — OUR, — CER, • RQ and d) On-line data for — pH level, in a batch-feeding fermentation process to produce Fab' at 450 L scale. Arrows indicate additions of lactose.

Results at 450 L scale indicated total Fab' levels of 75 mg/L, which represents 0.0018 mg of Fab' L^{-1} per g of lactose based on consumption of 41,280 g of lactose. This value was found to be ~95% less when compared to a total Fab' yield of 35 mg/L at 20 L scale, which represents 0.055 mg of Fab' L^{-1}per g based on consumption of 625 g of lactose. Results at pilot scale strengthened the hypothesis that O_2 limitation could limit product formation as the culture at this scale observed longer periods of time with DOT levels near to zero and less product formed per amount of lactose added compared to that at 20 L scale.

4.3.2. Scale-up analysis

Scale-up analysis was performed in order to estimate the operating conditions at 450 L scale from 20 L experiments. The comparison between scales helped to identify possible process variables that might be limiting product formation.

As a first step of the scale up analysis, the P_g/V was estimated from empirical engineering correlations only to set-up starting operating conditions for the 450 L fermenter. The criterion was to keep similar initial K_La values to the 20 L scale. Once fermentations were running, the K_La was determined based on on-line data (as discussed in section 3.2.8). Finally, estimation of mixing times, time of oxygen consumption and, time of oxygen transfer was performed as described in section 3.3. The following sections discuss each of these aspects in turn.

4.3.2.1 COMPARISON OF GASSED POWER PER UNIT VOLUME (Pg/V) AT 20 L AND 450 L SCALE

The gassed power at the beginning and at the end of the fermentation is summarised in table 4.2. As can be seen, the Reynold's number (Re) is above 10,000 in both processes and consequently a turbulent flow can be assumed (Bailey et al, 1986).

Table 4.1. Reynold's number (initial and final) in a Fab' fermentation process at 20 L and 450 L scale

Parameters	Initial conditions		Final conditions	
	20 L	450 L	20 L	450 L
Working volume (L)=	11	300	14	350
Working volume (m^3)=	0.011	0.3	0.014	0.35
δ (kg/m^3)=	1000	1000	1000	1000
μ (Pa*s)=	0.001025	0.001025	0.001025	0.001025
N (rpm)=	500	200	1200	500
N (rps)=	8.33	3.33	20	8.33
D_i (m)=	0.074	0.2	0.074	0.2
W_i (m)=	0.0015	0.04	0.0015	0.04
H_L (m)=	0.448	1.28	0.448	1.28
T (m)=	0.21	0.588	0.21	0.588
Re= $(\delta N D_{impeller}^2)/\mu$	44,520	130,081	106,849	325,203

where δ= density, μ= viscosity, N= agitation rate, D_i= impeller's diameter, W_i= impeller's width , H_L= liquid height, T= tank diameter, Re= Reynold's number

Based on empirical correlations (Bailey et al, 1986) the gassed and ungassed power was estimated. Results shown in table 4.3 indicated an 8-fold and a 10.5-fold increase in the gassed power from the beginning to the end of the process at 20 L and 450 L scale, respectively.

The difference of gassed power from the beginning to the end of the process between scales was mainly due to the cascade control which increased the agitation speed (and electric power required) in order to maintain the set level of DOT during the experimental growth phase.

Table 4.2. The gassed power (initial and final) in a Fab' fermentation process at 20 L and 450 L scale.

Parameters	Initial conditions		Final conditions	
	20 L	450 L	20 L	450 L
V (L)=	11	300	14	350
P_{NO}= f(Re)	5.7	5.7	5.7	5.7
$n_{impeller}$ =	3	3	3	3
Q(Lpm) =	2.5	100	15	450
Q(vvm) =	0.2	0.3	1.07	1.29
Q(m^3/s) =	0.00004	0.00167	0.00025	0.00750
P_{ug} (watt) = $P_{NO}\delta n_{impeller}N^3D_{impeller}^5$	21.96	202.67	303.56	3166.67
P_g (watt) = $0.72[P_{ug}^2ND_{impeller}^3)/Q^{0.56}]^{0.45}$	11.39	84.18	114.29	1033.04
P_g/V (kwatt/m^3) =	1.04	0.28	8.16	2.95

where V= working volume, P_{NO}= power number, $n_{impeller}$= number of impellers, Q= air flow rate, P_{ug}= power under ungassed conditions, P_g= power under gassed conditions

Some common values of P_g/V at production scale have been reported between 2-4 kW/m^3 (Sweere et al, 1987). Values estimated at 450 L scale were within the range of production scale figures. Nevertheless, values obtained at 20 L scale are out of the range reported by 2-fold. Therefore, increase of agitation rate and/or air flow, could not be a feasible DOT control strategy at large scale.

4.3.2.2 COMPARISON OF VOLUMETRIC MASS TRANSFER COEFFICIENT (K_La) AT 20 L AMD 450 L SCALE

The volumetric mass transfer coefficient at the beginning of the fermentation is presented in table 4.3. As described in section 3.3, initial K_La was estimated based on an empirical correlation; as the method described in section 3.2.8 could not be accurate enough due to the small amount of oxygen consumed by the cells and the relatively large amount of dissolved oxygen present in the broth at the beginning of the fermentation. Nevertheless, as can be observed in figure 4.3, stable readings of K_La ~400 h^{-1} were obtained until maximum values of agitation rate and air flow rate were reached. This is congruent with the strong dependence that K_La has with agitation rate, principally.

Table 4.3. Gassed power (initial and final) in a Fab' fermentation process at 20 L and 450 L scale.

Parameters	Initial conditions		Final conditions	
	20 L	450 L	20 L	450 L
P_g/V (kwatt/m^3)=	1.04	0.28	8.16	2.95
V_{gs} (m/s)= $Q/(\pi D_i^2/4)$	0.010	0.053	-	-
K_La (s^{-1})*= $0.026(P_g/V)^{0.4}V_{gs}^{0.5}$	0.041	0.057	-	-
K_La (h^{-1})=	148.05	205.524	-	-
Experimental K_La (h^{-1})=			400	300

where Pg/V= volumetric gassed power, V_{gs}= gas superficial velocity, K_La= volumetric mass transfer coefficient, D_i= impeller's diameter

Towards the end of the fermentation process at 450 L scale, values of K_La were dropping possibly due to the effect of cell lysis observed in the culture. In parallel the agitation rate was reduced as a consequence that the demand of oxygen was decreasing.

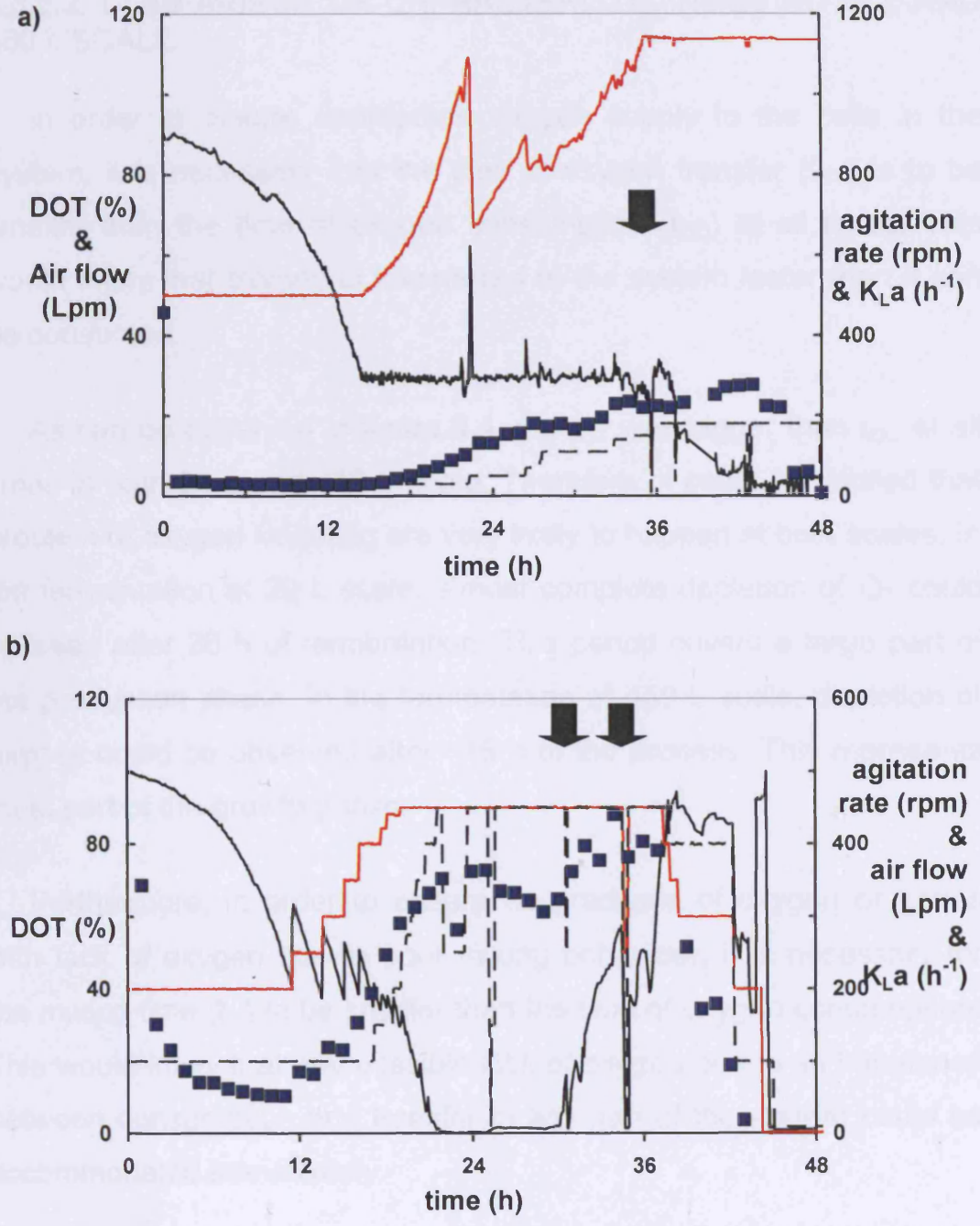


Figure 4.3 Comparison of -■- volumetric mass transfer coefficients (K_La), — DOT, ---- air flow and — agitation rate at a) 20 L scale and b) 450 L scale, to produce Fab' with cascade control and no gas blending. Arrows indicate addition of lactose.

4.3.2.3. COMPARISON OF CHARACTERISTIC TIMES AT 20 L AND 450 L SCALE

In order to ensure appropriate oxygen supply to the cells in the system, it is necessary that the time of oxygen transfer (t_{OT}) is to be smaller than the time of oxygen consumption (t_{OC}) at all times. This would imply that oxygen is transferred to the system faster than it can be consumed.

As can be observed in figure 4.4, the t_{OT} was bigger than t_{OC} at all times in both 20 L and 450 L scale. Therefore, it could be implied that problem of oxygen limitation are very likely to happen at both scales. In the fermentation at 20 L scale, almost complete depletion of O_2 could be seen after 36 h of fermentation. This period covers a large part of the production phase. In the fermentation at 450 L scale, depletion of oxygen could be observed after ~18 h of the process. This represents most part of the growth phase.

Furthermore, in order to ensure no gradients of oxygen or zones with lack of oxygen due to poor mixing behaviour, it is necessary for the mixing time (t_m) to be smaller than the time of oxygen consumption. This would imply that any possible lack of oxygen due to an imbalance between consumption and transfer in any part of the system could be accommodated immediately.

Minimum mixing time was evaluated as described in section 3.3 under maximum agitation rate. As can be seen in figure 4.4, it is not very likely to have mixing problems at 20 L scale. This is congruent with the fact that small scale fermentations usually present homogeneous environments (Sweere et al, 1987). On the other hand, t_m was found to be similar to t_{OT} but larger than t_{OC} by 10 seconds at 450 L scale. Therefore, it is quite possible to have zones with lack of oxygen due mainly to the high consumption of oxygen by the cells.

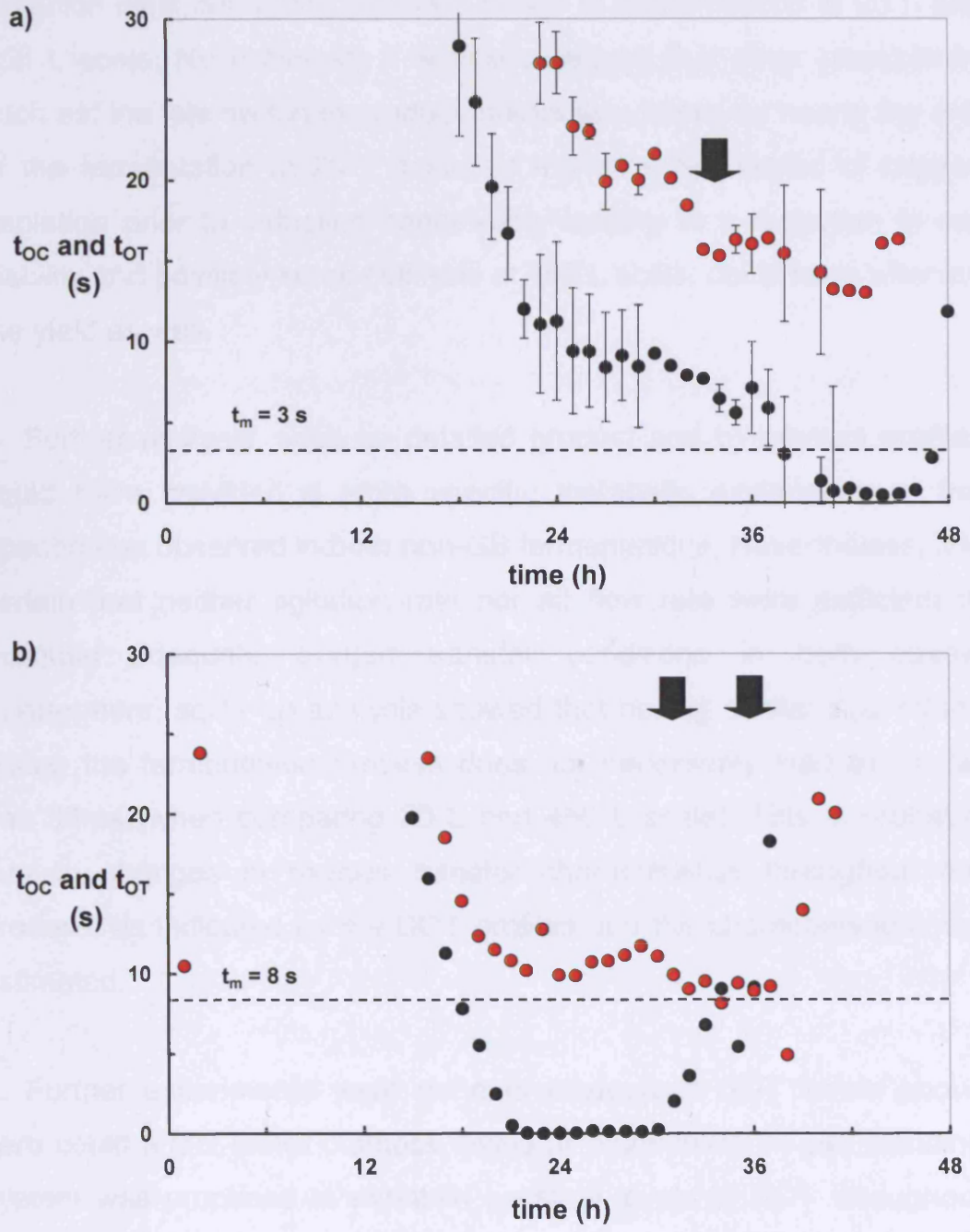


Figure 4.4 Comparison of • time for O_2 consumption, • time for O_2 transfer and minimum mixing time (t_m) at a) 20 L scale and b) 450 L scale, to produce Fab' with cascade control and no gas blending. Arrows indicate addition of lactose. Error bars represent standard deviations of duplicate fermentations.

Based on this analysis, it could be strongly suggested that oxygen limitation exist during the induction phase in fermentations at 20 L and 450 L scale. Nevertheless, it is acknowledged that other possibilities such as: the late switch to product metabolism observed nearly the end of the fermentation at 20 L scale, or the extended period of oxygen depletion prior to induction conceivably leading to a reduction in cell viability and possibly some cell lysis at 450 L scale, could have affected the yield as well.

Further analysis, such as detailed product and by-product profiles could have provided a more specific metabolic explanation of the phenomena observed in both non-GB fermentations. Nevertheless, it is certain that neither agitation rate nor air flow rate were sufficient to maintain adequate oxygen transfer conditions in both cases. Furthermore, scale up analysis showed that having similar K_La values during the fermentation process does not necessarily lead to similar Fab' titres (when comparing 20 L and 450 L scale). This is probably due to changes in oxygen transfer characteristics throughout the process, as indicated by the DOT profiles and the characteristic times estimated.

Further experiments were done to evaluate if DOT levels above zero could affect either biomass levels or productivity. A gas blending system was proposed to maintain constant levels of DOT throughout the whole fermentation process. The hypothesis that DOT levels have an effect on Fab' yields was tested and discussed in chapter 5.

Chapter 5

Characterisation of fermentation process in a Gas blending system

5.1. ABSTRACT

Fermentations carried out at 450 L and 20 L scale to produce Fab' antibody fragments indicated a serious problem to control levels of dissolved oxygen in the broth due to the high cell density and large oxygen demand. Dissolved oxygen tension (DOT) dropped to zero during the induction phase and it was hypothesised that this could limit product formation due to inadequate oxygen supply in the culture.

A gas blending system at 20 L scale was employed to address this problem and a factorial 2^2 experimental design was executed to evaluate independently the effects and interaction of two main engineering factors: agitation rate and DOT level (both related to mixing and oxygen transfer in the broth) on Fab' yields. By comparison to the non-gas blending system at 20 L, results in the gas blending system at same scale showed an increase in the production of Fab' by 77 % at an agitation rate of 500 rpm and independent of the DOT level and by 50 % at 1000 rpm with 30 % DOT. By contrast, fermentation culture at 1000 rpm with 50 % DOT showed no significant difference of Fab' yield compared to the non-gas blending system. Product localisation in the cell periplasm of > 90% was obtained in all fermentations. Results obtained encourage further studies at 450 L scale initially, to evaluate the potential of gas blending in the industrial production of Fab' antibody fragments.

5.2. INTRODUCTION

Oxygen availability is very critical for the aerobic growth of *E. coli* in fermentations and, at the same time, difficult to achieve, due to the poor solubility of oxygen (Gupta et al, 2003). In industrial fed-batch fermentations production often begins with an exponential feed phase of a limiting carbon source, until the limit of adequate oxygen transfer is reached (Sanden et al, 2003).

Gas blending has been proposed as a technique to address the problems of inadequate oxygen supply. Flores et al, 1994 reported that this led to increases in product yields for xanthan gums during cultivation of *Xanthomonas campestris* where the proportion of high molecular weight polymers was higher as DOT levels were increased. Changes in agitation and shear rate are also linked to oxygen mass transfer rates and consequently it is not always possible to identify the independent effect of oxygen levels on the fermentation, and the levels of metabolite production achieved. For example, *Aurebasidium pullulans* was found to be sensitive to high levels of oxygen independent of the agitation rate, and an increase of polysaccharide produced was possible by maintaining a low level of DOT at 1000 rpm (Gibbs et al, 1996).

In another study gas blending was used to separate the effects of DOT from agitation in a fungal fermentation for the production of pneumocandis. In this case the effect of dissolved oxygen was independent of agitation rate within a power range of 2-15kW/m^3, helping to define operating boundary conditions for successful scale up to 19m^3 (Pollard et al, 2002).

Also, the effect of agitation on the interaction between the extent of mixing in *Xanthan* fermentation broths and the rate of oxygen transfer was investigated. Findings showed that the biological performance of the culture was independent of agitation rate as long as broth homogeneity could be assured and that critical values of DOT of 6 to 10% were maintained in the production phase (Amanullah et al, 1998).

Information on the effect of DOT on recombinant protein production by *E. coli* is relatively scarce and no general rules can be derived. DOT has been shown to have variable effects on the production of different recombinant proteins. For example, Li et al, 2002 examined four recombinant strains of *E. coli* for the effects of the dissolved oxygen level on the level of biomass, the plasmid content, and the level of two level protein, i.e. chloramphenicol acetyltransferase and beta-galactosidase. The optimal dissolved oxygen concentration for the specific activity of recombinant proteins was found to be dependent upon the strain. In another study (De Leon et al, 2003) 1L bioreactors were used to determine the effect of DOT on the production of penicillin acylase by *E. coli*. Maximum activity was obtained at 1% DOT. In another work Bhattacharya et al, 1997 studied the overexpression of a target protein (MspI methylase) in recombinant *E. coli*. Results indicated that under oxygen-deficient conditions, the level of target protein decreased drastically.

This study is concerned with increasing the production titres of antibody fragments through control of DOT levels. The effect of DOT levels and agitation rates on Fab' production by *E. coli* at 20 L scale has been examined.

The principal aim was to design and carry out experiments to test the hypothesis that increased Fab' titres can be achieved in a fermenter system that is not oxygen limited. The questions tackled by this research have been addressed by comparison of a batch-fed fermentation of an oxygen deprived culture with those growing in environments with constant DOT levels of 30% or 50%.

In particular, the effect of DOT and agitation rate on growth kinetics, Fab' yield and cellular localisation were determined. Furthermore, the interaction between operating parameters and cell productivity has been evaluated by using statistical design of experiments (DoE). Gas blending has been an important tool to distinguish the effect of dissolved oxygen from agitation (Gibbs et al, 1996; Li et al, 1992) and together with scale-up studies and design of experiments is hoped to represent a valuable approach to evaluate this processes.

5.3. RESULTS AND DISCUSSION

5.3.1. Fermentation development

5.3.1.1 CHARACTERISATION OF THE PROCESS WITH GAS BLENDING

As described in the previous chapter, both fermentations at 20 L and 450 L scale run without gas blending had similar initial K_La's and were operated under cascade control to maintain a DOT level of 30%. Nevertheless, in both cases the DOT level dropped close to zero before induction. By contrast in fermentations which employed gas blending a constant level of DOT (ca. 30%) was maintained throughout the fermentation (see figure 5.1).

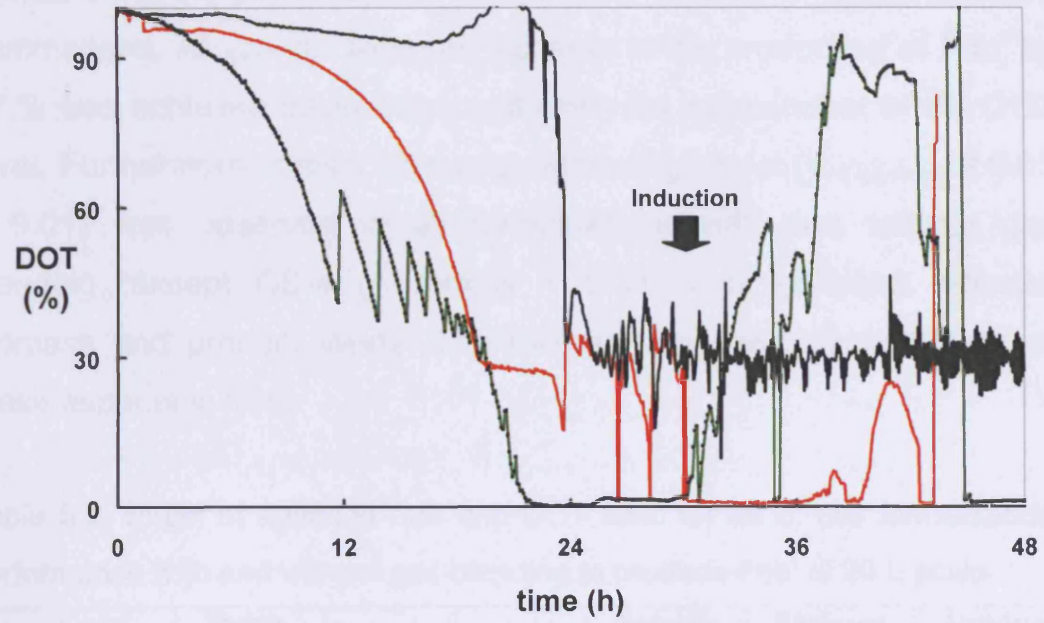


Figure 5.1. On-line DOT profiles of Fab' antibody fragment fermentation in a — 450 L scale with no gas blending and two 20 L scale fermentation — with and — without gas blending. The arrow indicates the point of induction.

The set of experiments presented in this chapter focused on the comparison of fermentations with and without gas blending at 20 L scale to allow the effect of gas blending on final Fab' yield, growth kinetics and cellular localisation of the product to be identified. Furthermore, effect of mixing and oxygen transfer on biomass levels and product yields was evaluated using a factorial design 2^2. As detailed in table 5.1, four fermentations with different O_2 transfer environments were set and analysed.

Table 5.1 Factorial experiment 2^2 to evaluate the effects and interactions of DOT level and agitation rate on Fab' yields

Exp. #	A = agitation rate (rpm)	B = DOT (%)
GB-1	-	-
GB-2	+	-
GB-3	-	+
GB-4	+	+

A= - (500rpm), + (1000rpm); B= - (30%), + (50%)

In table 5.2 the performances of these five different fermentations are summarised. As can be seen, an increase in the production of Fab' by 77.% was achieved using a low agitation rate independent of the DOT level. Furthermore, similar biomass yields on glycerol ($Y_{X/glycerol}$ of 0.35 ± 0.01) was observed in all fermentations with and without gas blending, except GB-4 (+ rpm & + DOT) that exhibited reduced biomass and product yields. The following sections discuss each of these aspects in turn.

Table 5.2. Effect of agitation rate and DOT level on an *E. coli* fermentation performance with and without gas blending to produce Fab' at 20 L scale

EXP	DCW (g/L)	Extra-cellular protein conc. (mg/L)	Final acetate conc. (g/L)	Fab' yield (mg/L)	Specific oxygen uptake rate[1] ($g/g.h^{-1}$)	Biomass yield on glycerol $Y_{X/glycerol}$[2] (g/g)	Product yield on lactose $Y_{P/lactose}$[3] (mg/g)
Non-GB	32.0	28.8	2.82	28.3	3.1	0.35	0.57
GB-1	30.6	24.6	2.23	134	9.7	0.34	2.68
GB-2	31.0	33.39	2.72	71.18	8.1	0.34	1.4
GB-3	30.6	23.4	1.01	133.5	9.8	0.34	2.67
GB-4	23.5	27.6	5.2	23.7	4.2	0.26	0.47

Total volume of 12.5 L was considered

1. Maximum OUR after induction was used to estimate the values of specific oxygen uptake rates.
2. A total amount of 1125 g of glycerol was used (including initial concentration plus additions).
3. A total amount of 625 g of lactose was added.

5.3.1.2 EFFECT OF GAS BLENDING ON BIOMASS LEVELS

A maximum specific growth rate of 0.17 +/- 0.02 h^{-1} was obtained for all fermentations with and without gas blending. As illustrated in figure 5.2, biomass levels of ca. 30 g/L were obtained in all 20 L fermentations except for that when the most extreme conditions of DOT and agitation speed were used (GB-4). For the 20 L fermentation GB-4 (+ rpm & + DOT) the biomass levels produced were 17% lower than the average level of all the other fermentations.

The lower level of biomass in GB-4 was unexpected and the protein concentration was therefore measured in the supernatant to determine whether the reduced biomass levels could be attributed to increased levels of cell lysis or shear damage under conditions of high agitation and aeration. Results presented in figure 5.3 showed that total protein values of 25 to 30mg/L where obtained in the supernatant of all five fermentations, effectively ruling out cell lysis as a reason for the differences in biomass levels.

All gas blending fermentations yielded higher maximum OUR levels than the non-gas blending system with 100 $mmol.L^{-1}h^{-1}$ when comparing the respiration profiles (see figure 5.4). Nevertheless, fermentation GB-4 (+ rpm & + DOT) observed the lowest levels of OUR values: 100-150 $mmol.L^{-1}h^{-1}$. This value was ~65% less compared to the OUR values determined in the other three gas blending fermentations. Furthermore, as can also be seen in figure 5.4, diauxic growth was observed in all fermentations except GB-4.

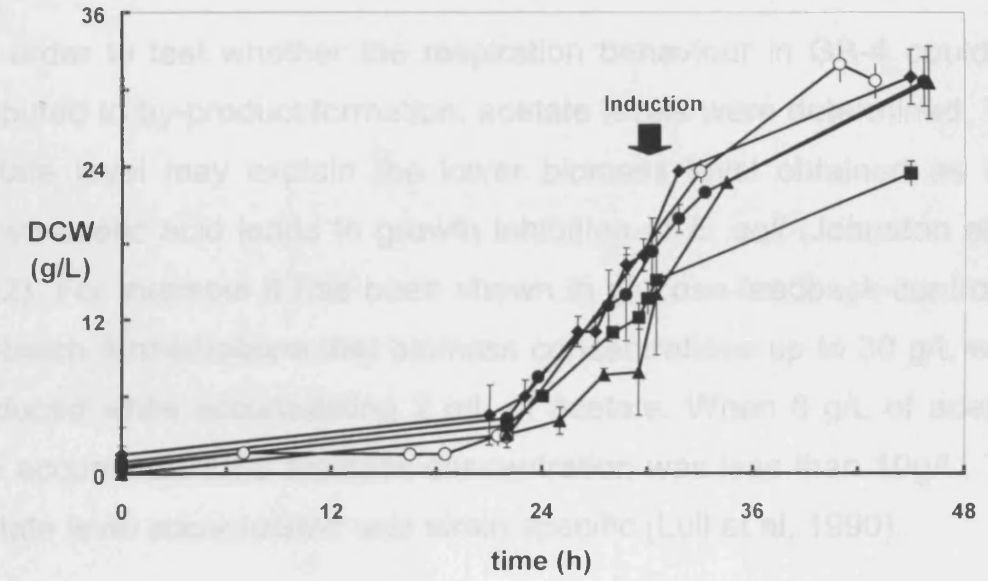


Figure 5.2 Effect of gas blending on final biomass levels in two different fermentation systems (from left to right): -○- 20 L non gas blending, 20 L gas blending from run 1 to 4 according to the 2^2 factorial design of experiments (see table 5.1): -●- GB-1 (-rpm, -DOT), -◆- GB-2 (+rpm, -DOT), -▲- GB-3 (-rpm, +DOT), -■- GB-4 (+rpm, +DOT). Error bars represent standard deviations of duplicate fermentations.

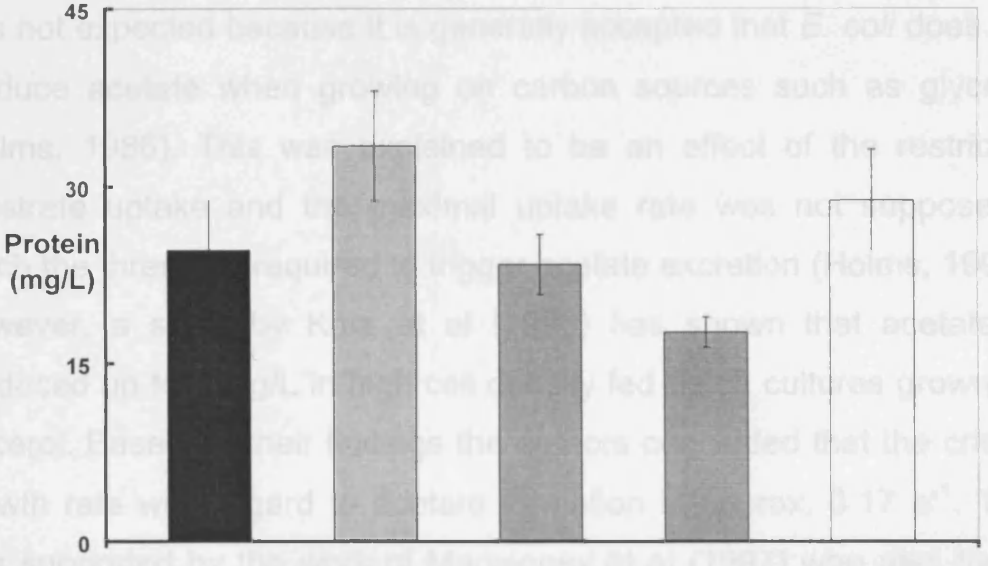


Figure 5.3 Effect of gas blending on final protein levels in two different fermentation systems at 20 L scale with (GB) and without gas blending (non-GB) (from left to right): -■- GB-1 (-rpm, -DOT), -▫- GB-2 (+rpm, -DOT), -▪- GB-3 (-rpm, +DOT), -▪- GB-4 (+rpm, +DOT); and -▫- non-GB. Error bars represent standard deviations of duplicate fermentations.

In order to test whether the respiration behaviour in GB-4 could be attributed to by-product formation, acetate levels were determined. The acetate level may explain the lower biomass level obtained as it is known acetic acid leads to growth inhibition of *E. coli* (Johnston et al, 2002). For example it has been shown in glucose-feedback-controlled fed-batch fermentations that biomass concentrations up to 30 g/L were produced while accumulating 2 g/L of acetate. When 8 g/L of acetate was accumulated the biomass concentration was less than 10g/L. The acetate level accumulated was strain specific (Luli et al, 1990).

As can be seen in figure 5.5, GB-4 (+ rpm & + DOT) exhibited the highest concentration of acetate at the end of the fermentation. Although cycles of excretion and re-consumption could be expected throughout the fermentation, it is an indication that the acetate levels reached in GB-4 most probably where higher than those of the other fermentations. The excretion of acetate under fully aerobic conditions was not expected because it is generally accepted that *E. coli* does not produce acetate when growing on carbon sources such as glycerol (Holms, 1986). This was explained to be an effect of the restricted substrate uptake and the maximal uptake rate was not suppose to reach the threshold required to trigger acetate excretion (Holms, 1996). However, a study by Korz et al (1996) has shown that acetate is produced up to 3.3 g/L in high cell density fed batch cultures grown on glycerol. Based on their findings the authors concluded that the critical growth rate with regard to acetate formation is approx. 0.17 h^{-1}. This was supported by the work of Macaloney et al (1997) who also found acetate excretion in fed-batch cultures of *E. coli* grown on glycerol when a critical specific growth rate was exceeded. Since the specific growth rates obtained in all GB and non GB fermentations are very close to the reported threshold value the excretion of acetic acid during the growth phase is likely.

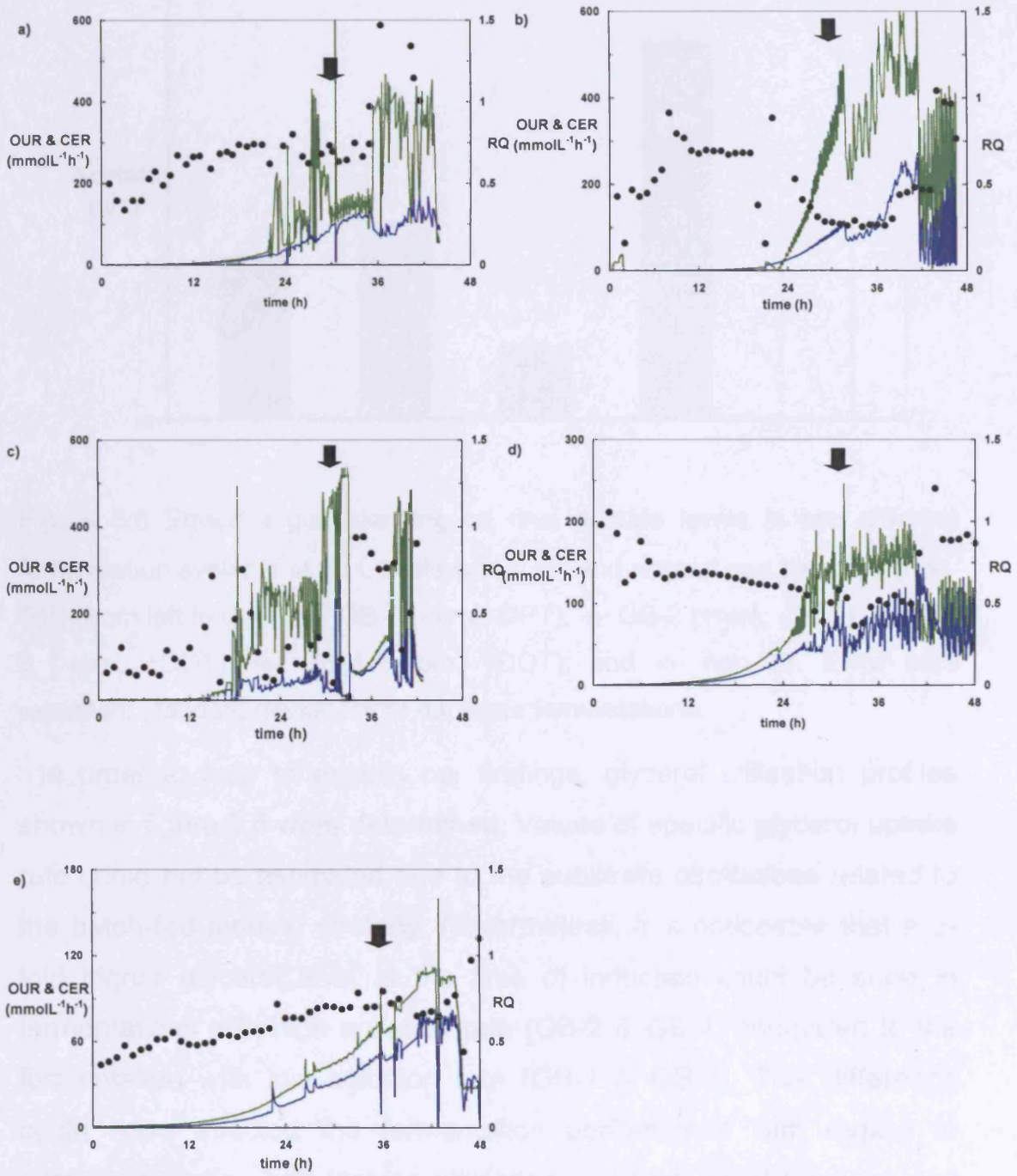


Figure 5.4. Effect of gas blending on OUR and CER in two different fermentation systems at 20 L scale with gas blending (GB) and without gas blending (non-GB): a) GB-1 (-rpm, -DOT), b) GB-2 (+rpm, -DOT), c) GB-3 (-rpm, +DOT), d) GB-4 (+rpm, +DOT); and e) non-GB. Arrow indicates induction.

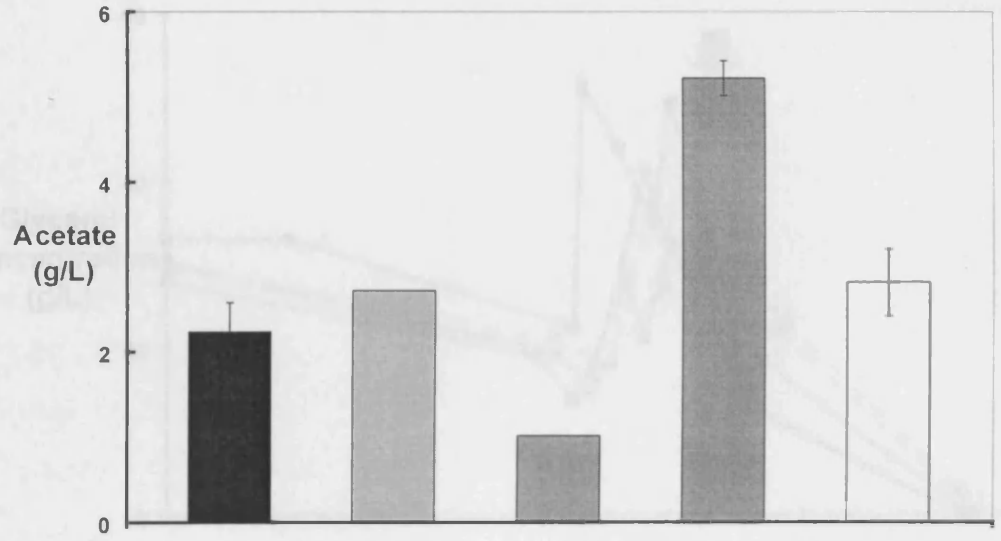


Figure 5.5 Effect of gas blending on final acetate levels in two different fermentation systems at 20 L scale with (GB) and without gas blending (non-GB) (from left to right): -■- GB-1 (-rpm, -DPT), -□- GB-2 (+rpm, -DOT), -■- GB-3 (-rpm, +DOT), -▣- GB-4 (+rpm, +DOT); and -□- non-GB. Error bars represent standard deviations of duplicate fermentations.

In order to help to explain our findings, glycerol utilisation profiles shown in figure 5.6 were determined. Values of specific glycerol uptake rate could not be estimated due to the substrate oscillations related to the batch-fed feeding strategy. Nevertheless, it is noticeable that a 2-fold higher glycerol level at the time of induction could be seen in fermentations with high agitation rate (GB-2 & GB-4) compared to the fermentation with low agitation rate (GB-1 & GB-3). This difference could have affected the fermentation performance with regard to acetate formation and lactose utilisation and thus could have caused an additional strain on the cells. It has been reported that acetate is produced when carbon flux exceeds the biosynthetic demands of the capacity for energy generation within the cell in a glucose-based system (El-Mansi et al, 1989; Lee, 1996; Rocha et al, 2002). Therefore, a similar phenomenon in this glycerol-based system could have occurred and the carbon flux was exceeded in GB-2 and GB-4 due to the high mass transfer as a result of the higher agitation rate.

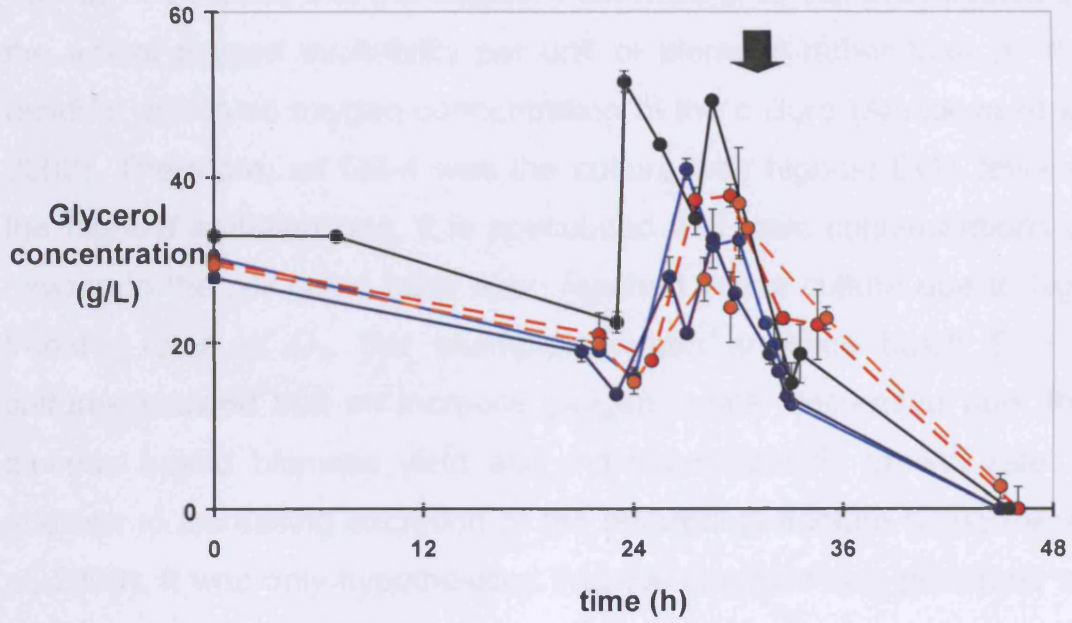
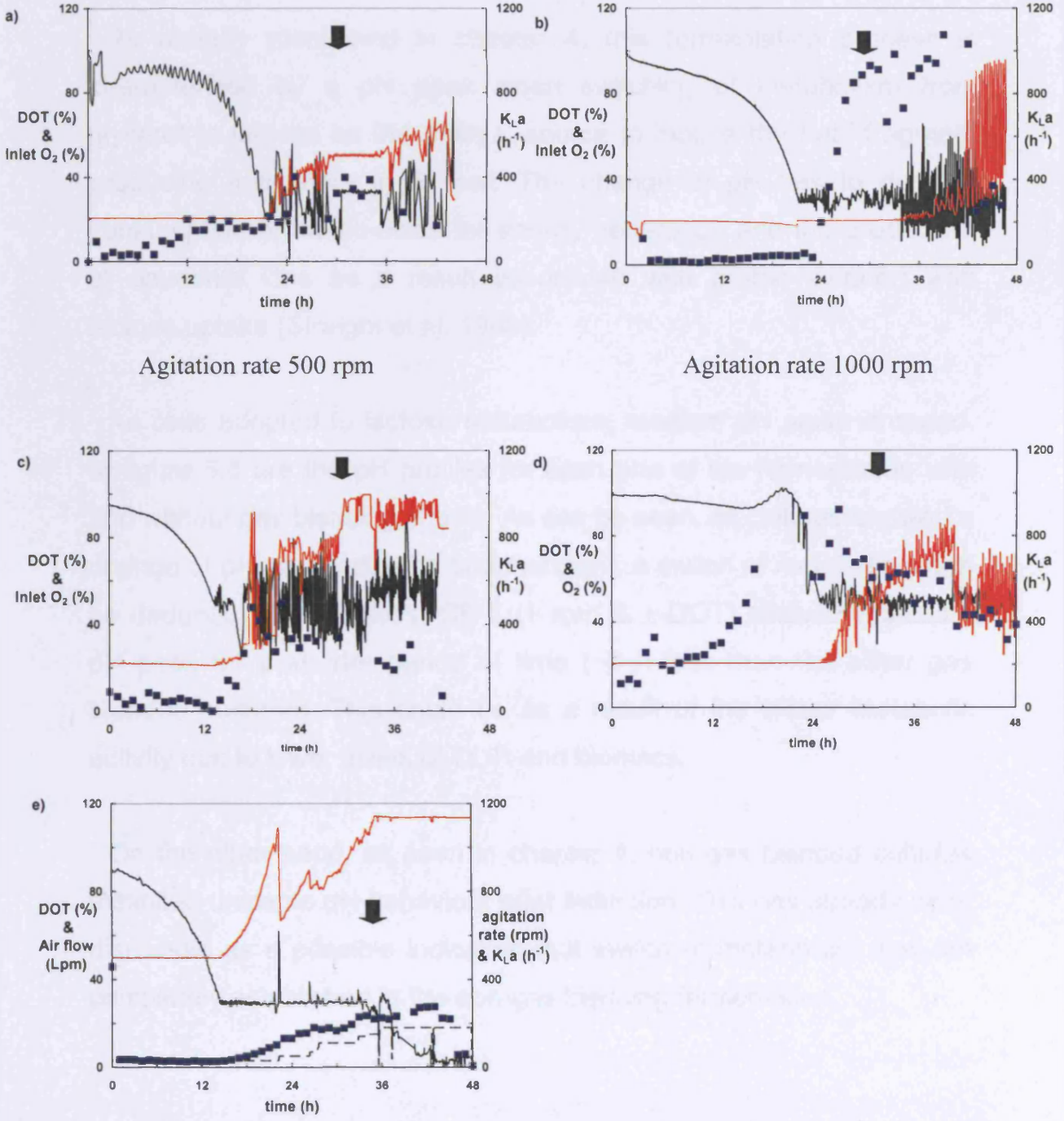


Figure 5.6 Effect of gas blending on glycerol uptake rate in two different fermentation systems at 20 L scale with (GB) and without gas blending (non-GB) (from left to right): GB-1 (-rpm, -DOT), GB-2 (+rpm, -DOT), GB-3 (-rpm, +DOT), GB-4 (+rpm, +DOT); and non-GB. Arrow indicates induction. Error bars represent standard deviations of duplicate fermentations.

On the other hand, acetate level in non-GB fermentation was similar to that observed in GB-2 despite using gas blending. Furthermore, the acetate level in GB-2 was 47 % lower to that in GB-4. This last result suggests that oxygen has a significant effect on acetate formation, as the main difference between GB-2 and GB-4 is the DOT level. It is known that high concentrations of oxygen can be toxic, highly reactive and can spontaneously cause unwanted oxidation reactions within the cell (Lodish et al, 2000). On the other hand, levels up to 100% air saturation in *E. coli* fermentations using DOT control have been reported with no inhibitory effect on biomass formation (Li et al, 1992). All fermentations in this work used oxygen levels up to 90% air saturation to maintain a constant DOT of 30 or 50%. Nevertheless, GB-4 (+ rpm & + %DOT) observed the highest concentrations of dissolved oxygen at the highest agitation rate.

It has been found that the catabolic activities of *E. coli* are affected by the actual oxygen availability per unit of biomass rather than by the residual dissolved oxygen concentration of the culture (Alexeeva et al, 2002). Therefore, as GB-4 was the culture with highest DOT level at the highest agitation rate, it is speculated that toxic concentrations of oxygen in the cell could have been reached in this culture due to high transfer rates of O_2. For example, oxygen enriched batch *E. coli* cultures showed that an increase oxygen supply decreased both the glucose based biomass yield and maximum specific growth rate in addition to increasing excretion of the by-product acetate (O'Beirne et al, 2000). It was only hypothesised that the intermediates produced as a result of the reduction of an oxygen molecule were themselves toxic. Therefore, further work is needed to evaluate whether similar behaviour is observed when glycerol is used as the main C-source and a mechanistic explanation can be proposed.

All fermentations in this work used oxygen levels of up to 90 % of air saturation to maintain a constant DOT of 30 or 50 % (see figure 5.7). Nevertheless, GB-4 (+ rpm & + %DOT) had the highest concentrations of dissolved oxygen at the highest values of agitation rate. In order to determine the difference of oxygen transfer rates between fermentations, K_La values were calculated from respiration profiles (see figure 5.7). A maximum value of ~800 h^{-1} was estimated in the two gas blended cultures at high agitation rate, regardless of the DOT level. This value was found to be ~50 % higher compared to non-gas blending system (K_La= ~400) and the other two gas blending cultures at low agitation rate (K_La=~300), respectively. This is in agreement with the fact that K_La is strongly dependant on agitation rate more than the O_2 concentration in the broth. Further work is needed to verify if this high mass transfer rates of nutrients and oxygen under high agitation rate and high DOT conditions could effectively cause toxic O_2 levels within the cells in GB-4.



Agitation rate 500 rpm

Agitation rate 1000 rpm

Figure 5.7. Effect of gas blending on -■- volumetric mass transfer coefficients (K_La), — DOT, — inlet O_2 concentration at 20 L scale in: a) GB-1 (-rpm, -DOT), b) GB-2 (+rpm, -DOT), c) GB-3 (-rpm, +DOT), d) GB-4 (+rpm, +DOT), e) non-GB. Arrow indicates induction.

5.3.1.3. EFFECT OF GAS BLENDING ON Fab' PRODUCTION

As already mentioned in chapter 4, this fermentation process is characterised by a pH peak when switching of metabolism from glycerol to lactose as the main C-source to induce the Fab' fragment producing machinery in *E. coli*. The change of pH has to do with consumption of amino-acids for energy generation and the production of ammonia ions as a result associated with proton symport with lactose uptake (Straight et al, 1988).

As cells adapted to lactose metabolism, medium pH again dropped. In figure 5.8 are the pH profiles for each one of the fermentation with and without gas blending shown. As can be seen, all cultures showed a change of pH after induction and therefore, a switch of metabolism can be deduced. Nevertheless, GB-4 (+ rpm & + DOT) showed a smaller pH peak for a shorter period of time (~3 h less than the other gas blended cultures). This could be as a result of the lesser metabolic activity due to lower levels of OUR and biomass.

On the other hand, as seen in chapter 4, non gas blended cultures exhibited unstable pH behaviour after induction. This has already been discussed as a possible indication that switch of metabolism was not completely established in the non-gas blending fermentation.

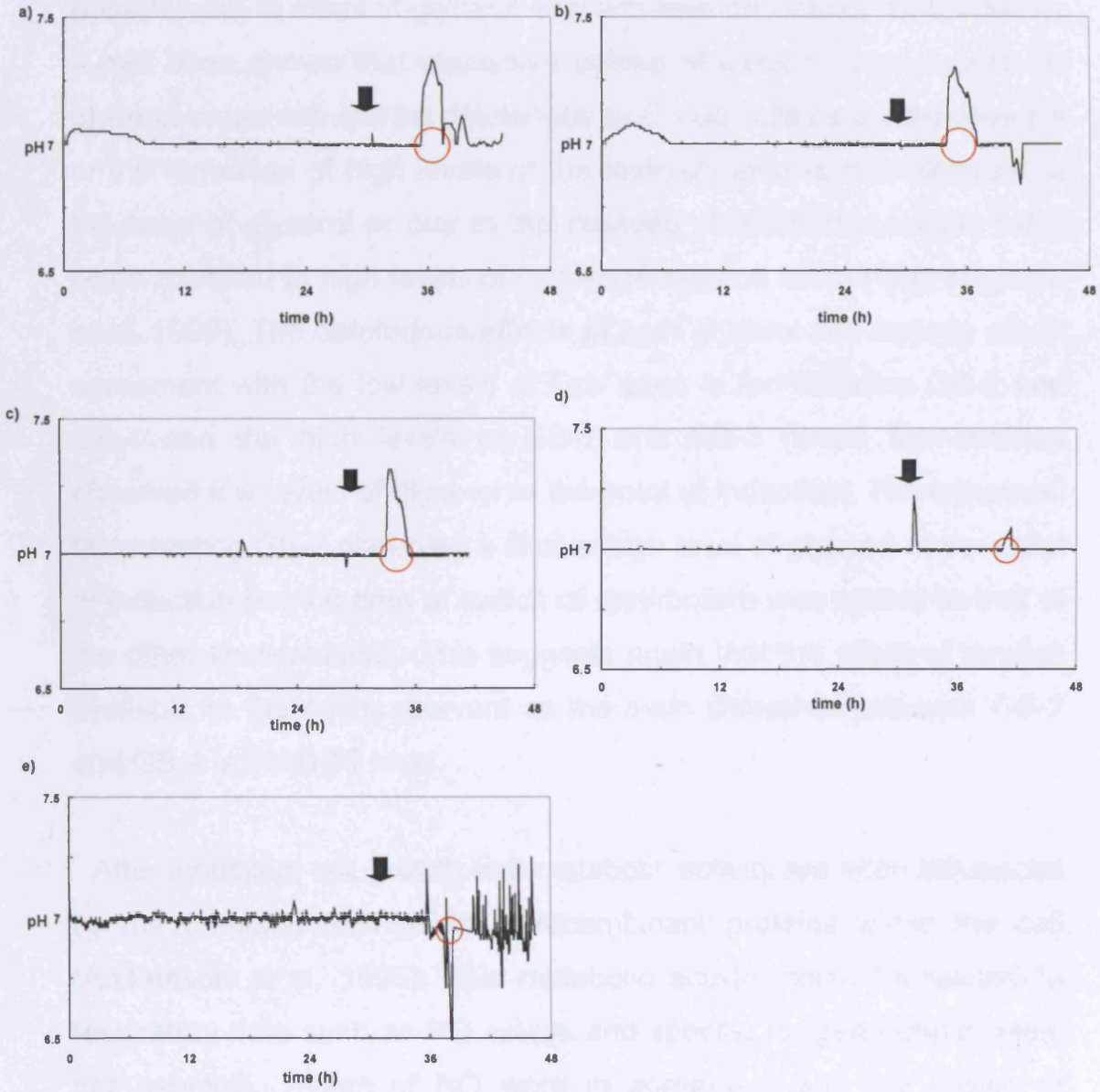


Figure 5.8 Effect of gas blending on pH profiles in two different fermentation systems at 20 L scale with gas blending (GB) and without gas blending (non-GB): a) GB-1 (-rpm, -DOT), b) GB-2 (+rpm, -DOT), c) GB-3 (-rpm, +DOT), d) GB-4 (+rpm, +DOT); and e) non-GB

Fermentation GB-4 observed the latest switch of metabolism (~40 h), possibly due to effect of glycerol concentration on lactose consumption. It has been shown that excessive uptake of carbon sources such as glycerol or lactose can be deleterious to *E. coli* cells as a consequence of the formation of high levels of the toxic compound methylglyoxal in the case of glycerol or due to the collapse of the proton motive force when exposed to high levels of lactose (Ferguson et al, 1998; Hogema et al, 1999). The deleterious effects of both glycerol and lactose are in agreement with the low levels of Fab' seen in fermentation GB-2 and GB-4 and the high levels of GB-1 and GB-3 (these fermentation observed low levels of glycerol at the point of induction). Nevertheless, fermentation GB-2 observed a similar high level of glycerol at the point of induction but the time of switch of metabolism was similar to that of the other fermentations. This suggests again that the effect of oxygen available to the cell is relevant as the main difference between GB-2 and GB-4 is the DOT level.

After induction, cell growth and metabolic activity are often influenced by the on-going expression of recombinant proteins within the cell (Andersson et al, 1996). This metabolic activity could be related to respiratory data such as RQ values and specific oxygen uptake rates. For example, values of RQ were in agreement with the switch of metabolism. A switch of RQ ca. 0.7 to ca. 1 was observed at the same time as that of the pH peak in all fermentations. Furthermore, when comparing specific oxygen uptake rates obtained from off-gas analysis during the induction period (see table 3), values of ~12 mmoL/g.h^{-1} for both GB-4 and the non-GB fermentations with similar low Fab' yields were found.

By contrast, fermentations GB-1 and GB-3 that both yielded higher Fab' titres observed values of ~45 mmoL/g.h^{-1}. It appears that the higher the oxygen consumption is by the cells the higher are metabolic activity and therefore product formation. Nevertheless, at this point it is not possible to determine what percentage of oxygen is related to product metabolism as opposed to cell maintenance and therefore further work is needed to establish if a direct correlation between specific oxygen uptake rate and product yield exist.

In table 5.3 the effect of gas blending on product formation is summarised. The Fab' titres were measured at the time of harvest (~46 hrs) for all fermentations. From the results, it can be suggested that the high agitation rate had a negative effect regardless of the DOT level in the gas blending system. On the other hand, a 77 % increase in Fab' yield was achieved when comparing the gas blending system with low agitation rate with the non-gas blending fermentation.

Comparison of the final volumetric Fab' concentrations in Figure 5.9 shows that conditions of low agitation and low DOT (GB-1) and low agitation and high DOT (GB-3) resulting in 3 fold higher concentrations compared to those of the other fermentations. At 20 L without gas blending the fermentation ran out of oxygen after 30 hrs (i.e. during the induction period).

Table 5.3. Specific Fab' titres and the proportion of periplasmic expression as functions of scale and application of gas blending in three different fermentation systems: 20 L without gas blending, 20 L gas blending (GB-1 to GB-4) according to the 2^2 factorial DoE (see table 5.1).

Fermentation	Fab' titres (mg Fab'/g DCW)			
	Periplasm	Extracellular	Total	% retention in periplasm
20L without gas blending	0.9	0.7	1.6	56
GB-1 (low agitation & low DOT)	4.4	0.6	5.0	88
GB-2 (high agitation & low DOT)	1.9	0.4	2.3	83
GB-3 (low agitation & high DOT)	4.0	0.3	4.4	91
GB-4 (high agitation & high DOT)	1.5	0.1	1.6	94

In this case, expression of Fab' possibly occurred under conditions of low oxygen which might have limited product synthesis. In terms of economics, low agitation & low DOT (GB-1) is a less expensive system as it requires less oxygen while obtaining similar levels of Fab' and biomass as compared to low agitation & high DOT (GB-3). This will become an important consideration at large-scale. Furthermore, in all gas blending fermentations, the level of periplasmic product localisation was high, with > 85% being retained in the periplasm.

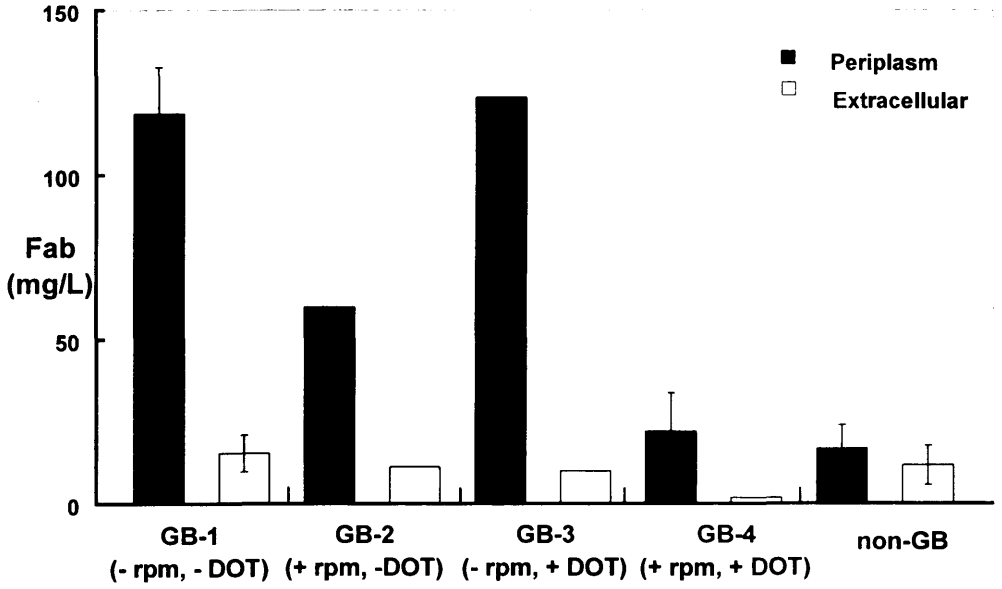


Figure 5.9. Final periplasmic and extracellular Fab' antibody fragment concentration in two different fermentation systems: 20L scale with gas blending according to the 2^2 factorial DoE (see table 5.1), and 20L scale without gas blending. Error bars represent standard deviations of duplicate fermentations.

5.3.1.4 STATISTICAL ANALYSIS

DoE methodology (Montogmery, 2001) allowed obtaining further information towards optimisation of this fermentation process. In table 5.4 the analysis of variance to evaluate the effects and interactions of DOT and agitation speed on Fab' production is summarised. It can be observed, that the effect of A (agitation speed) is significant for Fab' titres as the P value is 0.001. This would imply that there is 95 % probability that agitation rate has a large impact on Fab' yield when considering experimental error from duplicates. The effect of B (DOT) and its interaction with A appeared to be small relative to the main effect of A and seems to be not statistically significant ($P > 0.01$). Results suggest that from the two engineering factors studied, agitation rate is the main factor to focus on towards optimisation of this fermentation process. Nevertheless, further work is needed to evaluate different levels of agitation rate to study the effect on Fab' yields and by-product formation in more detail.

Table 5.4 Analysis of variance (ANOVA) to evaluate effects and interactions of DOT and agitation speed on the production of Fab' at 20 L scale in a gas blending system.

Factor	Coefficient	Std. Dev.	t3	P
A= Agitation	-42.13	3.64	11.57	0.001
B = DOT	-10.95	3.64	3.01	0.057
A*B	-8.62	3.64	2.37	0.099

where t_3 is the "t" test for the distribution F with 3 degrees of freedom and P is the probability to test in the system.

To summarise the ANOVA results, a linear model could be proposed from regression analysis of the data:

$$\text{Total Fab' (mg/L)} = 90.87 - 42.13N$$

where N is a coded variable that represents the agitation rate in rpm. The relationship between the natural variable and the coded one is as follows:

$$N = \frac{agitation - (agitation_{low} + agitation_{high})/2}{(agitation_{high} - agitation_{low})/2}$$

This model is a first approach towards optimisation of the process and indicates that agitation rate has an impact of 56 % on Fab' yields when changing agitation rate between 500 and 1000 rpm.

5.3.2. Effect of gas blending and agitation rate on characteristic times

It is well known that mass transfer conditions are strongly dependant on agitation rate in fermentation processes (Nielsen et al, 2003). Nevertheless, the gas blending effect should also be important, as it affects the mass transfer driving force by increasing the concentration of oxygen in the broth.

In the four gas blending fermentations performed, it was found that the main effect on characteristic times was due to the agitation rate. In figure 5.10, is shown a comparison between the two processes with the highest and the lowest yield, GB-1 and GB-4 respectively.

As can be seen, there is not a significant difference between time for oxygen consumption (t_{OC}) and time for oxygen transfer (t_{OT}). As mentioned in section 4.3.2.3 t_{OC} should be less than t_{OT} to ensure oxygen supply to the cells at all time. Therefore, it could be suggested that no oxygen limitation is likely to occur in the fermentations performed with gas blending, regardless of the agitation rate. This is clearer in the fermentation at high agitation rate.

On the other hand, when comparing the mixing times, the results suggest that it is possible to have mixing problem at low agitation rate (although unlikely at this small scale). As already mentioned in section 4.3.2.3, t_m should be less than t_{OC} to ensure no oxygen gradients. Nevertheless, at high agitation rate with gas blending, no mixing problems were observed. This result was expected due to effect of agitation rate on bubble size and consequently on the superficial contact area between the gas-liquid phase.

The results reported in this chapter suggest a fermentation operating strategy that employs gas blending can increase Fab' titres in bioreactor systems that are not oxygen limited when appropriate mass transfer conditions are achieved.

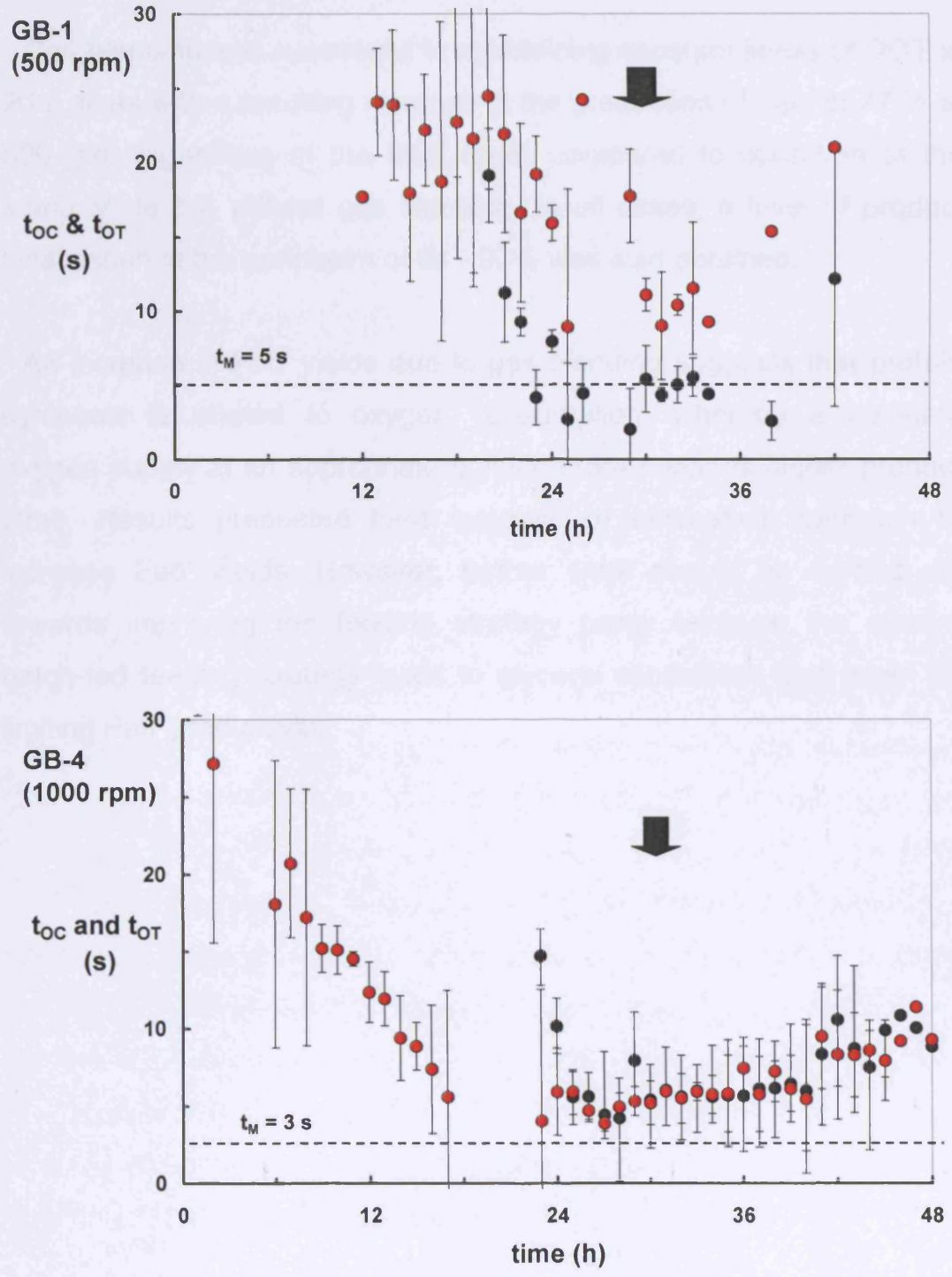


Figure 5.10 Effect of gas blending and agitation rate on • time for O_2 consumption (t_{OC}) and • time for O_2 transfer (t_{OT}) at a) GB-1 and b) GB-4, to produce Fab' in a non-gas blending system. Arrow represents induction. Error bars represent standard deviations of duplicate fermentations.

Gas blending was successful in maintaining constant levels of DOT at 20 L scale with a resulting increase in the production of Fab' of 77 % at 500 rpm regardless of the DOT level, compared to operation at the same scale but without gas blending. In all cases, a level of product localisation in the periplasm of 84 - 93% was also obtained.

An increase in Fab' yields due to gas blending suggests that protein synthesis is related to oxygen consumption, whereby a constant oxygen supply at an appropriate agitation rate supports higher product titres. Results presented here suggest an alternative approach to increase Fab' yields. However, further work should be carried out towards improving the feeding strategy partly because the current batch-fed feeding strategy leads to glycerol oscillations that might be limiting Fab' productivity.

Chapter 6

Characterisation of feeding strategies on a fermentation process in a Gas blending system

6.1. ABSTRACT

In order to achieve a higher cell density in a shorter period of time in this fermentation process, evaluation of temperature and concentration of nutrients on biomass levels was performed using a 2^2 factorial design in shake flasks. Results indicated that higher levels of nutrients could increase the final cell density. However, when transferred to 20 L scale in a gas blending system, growth inhibition was found, most likely due to high concentration of acetate (>7 g/L). Pulsed fed-batch (or batch-fed) fermentations carried out at 20 L scale, indicated presence of glycerol oscillations, therefore, a pH-stat feeding strategy was proposed. This pH-feeding strategy was implemented by direct coupling of growth and the related production of H^+, and this led to a steady state with a minimum constant concentration of glycerol (main C-source) in the induction period. This change of environment resulted in a 2-fold increase in the production of Fab' compared to those obtained using repeated batch strategy.

A glycerol yield coefficient ($Y_{X/glycerol}$ in $g_{cell}/ g_{glycerol}$) and product yield on biomass ($Y_{P/X}$ in $mg_{product}/g_{cell}$) of 0.39 and 5.91, respectively, were estimated for the pH-feeding fermentation. By contrast, $Y_{X/glycerol}$ and $Y_{P/X}$ were found to be 13 % and 45 % less, respectively, in the repeated batch process. Therefore, a better glycerol utilisation and product formation of the cells could be suggested in the pH-feeding fermentation.

Product localisation in the cell periplasm of >90 % was obtained in both processes. A difference of product loss in the centrifugation step of less than 1 % was observed between fermentations. Overall, a method to improve production of Fab' fragments without compromising primary recovery of cells by maintaining constant low levels of glycerol has been developed.

6.2. INTRODUCTION

A major objective of fermentation processes is to maximise the volumetric productivity. High cell density cultures are the first step in order to achieve this goal. However, production is limited by a multitude of factors.

Modelling of fermenter hydrodynamics and mixing, supported both by large scale measurement, and scale-down studies all point to the existence of concentration gradients even within moderate scale bioreactors (Bylund et al, 2000). Substrate oscillations have been reported to cause growth and product inhibition, even at low levels, due to imbalances in the carbon uptake rate and the switch from purely oxidative to fermentative (oxido-reductive) metabolism (Johnston et al, 2002). For example, studies by Lin et al, (2000) have indicated that glucose oscillations can influence product stability and the overgrowth of plasmid-free cells in the production of recombinant α-glucosidase by *E. coli.*

Substrate oscillations can be minimised by adopting appropriate feeding strategies, but a difficulty is that the critical substrate uptake rate often is poorly known and may even be time-variant (Akesson et al, 2001). Different feeding strategies designed to increase product yield in *E. coli* systems have been studied.

For example, the performance of a fermentation process to produce glutathione using a constant feed-rate, via a glucose feedback with manual adjustment of flow rate, and an exponential feeding strategy was compared (Li et al, 1998). Results suggested that exponential feeding was the best option for the system studied in terms of biomass levels up to 80g/L dry cell weight (DCW), but this strategy led to decreased level of biosynthesis and productivity of glutathione. Robbins et al (1989) proposed making glucose additions in a 12 L fermenter by taking advantage of the switch from acid to base formation reflected in the pH of the culture so as to optimise the growth of *E. coli* on protein hydrolysate (L-broth). Results indicated a doubling of cell densities and 31% cost savings based upon media cost per unit cell density achieved.

Chen et al (1997) reported on an *E. coli* fermentation process for the production of supercoiled plasmid DNA, using a computer-aided processing system to control interactively both the glucose feed-rate and agitation speed based on maintaining a constant level of dissolved oxygen. The proposed process was evaluated at 7 L and 80 L scales and resulted in 100 mg/L of product yield with a final biomass level of 60 g/L (dry cell weight). By contrast, the manual fed-batch process produced a low cell density ~10 g/L and product yield of ~8 mg/L.

In a recent study Kleist et al (2003) developed a control system to keep either the glucose concentration constant, or to maintain a low, but constant, oxygen level in a 7 L fermenter *E. coli* culture to produce phytase. Results indicated that high levels of activity and short cultivation times of 14 hrs could be achieved when the substrate level was held constant.

The study reported in this chapter is concerned with evaluating the effect of level of C-source on cell density. Studies at shake flask scale and at 20 L scale were carried out to evaluate the effect of nutrient concentration on final biomass levels.

A pH-stat feeding strategy was also evaluated to address the issue of glycerol oscillations that were observed in pulsed fed-batch *E. coli* fermentations. The pH-stat fermentation was chosen based on the principle that utilisation of C-source lead to acetate formation and NH_4^+ used to maintain the pH. Thus, the glycerol feed was coupled to the alkali supply which restores neutral pH in the fermentation broth. Fermentations were carried out in a gas blending system to maintain a constant level of DOT and adequate oxygen supply throughout the process. Gas blending has been reported in the previous chapter as a successful method for maximising productivity.

Furthermore, this study examines the potential impact of introducing a pH-stat feeding strategy on cell integrity and hence the potential for product loss in the subsequent centrifugation step for cell harvest. Since the downstream process can represent a significant percentage (50 to 80%) of the final cost of a product it is valuable to consider the impact of fermentation operating conditions on such recovery steps (Roque et al, 2004)

The Fab' is expressed into the periplasmic space of the *E. coli* host. Periplasmic expression compartmentalises the target protein so that it can be selectively released into a smaller process volume and free of many host cell protein and intracellular contaminants. As a direct consequence periplasmic expression also reduces the protein loading in later steps compared to other expression systems, e.g. intracellular expression (Pierce et al, 2002).

We were concerned to know whether changes in fermentation conditions might improve titres but also directly impact cell wall strength and lead to changes in product yields in early DSP. From a process perspective it is clearly desirable that any increase in titre is maintained trough the primary product capture stages.

The principal aim in this study was to test the hypothesis that increased Fab' titres can be achieved in a fermenter system under an appropriate feeding strategy with these improvements being maintained into primary recovery operation.

The hypothesis was addressed by comparison of the outputs of such pulsed fed-batch fermentations with those growing under a different concentration of glycerol and a pH-stat feeding strategy. The effect of the feeding strategy on growth kinetics, Fab' yield, cellular localisation and cell wall strength were determined as was the effect of such strategy on product loss occurring during centrifugation.

6.3. RESULTS AND DISCUSSION

6.3.1 Evaluation of nutrient concentration in the fermentation process

6.3.1.1 EFFECT OF NUTRIENT CONCENTRATION ON BIOMASS LEVELS IN SHAKE FLASKS

Fermentations at shake flask scale (250 mL working volume) were performed in order to evaluate the effect of nutrient concentration and temperature on growth kinetics. Both parameters are related to cell growth and therefore might have an effect on final biomass levels.

Shake flask cultures were run in a batch mode with no gas blending. In table 6.1 the set of experiments is summarised. Analysis of these experiments was the starting point to suggest an alternative feeding strategy for this Fab' fermentation.

Table 6.1. Factorial 2^2 model to evaluate effect and interactions between concentration of nutrients and temperature on growth kinetics in fermentations at 250 mL scale (shake flasks).

Fermentation	A= Nutrients Level (g/L)	B= Temperature
SF-1	-	-
SF-2	+	-
SF-3	-	+
SF-4	+	+

A= -(normal, e.g glycerol, 30g/L), +(double, e.g. glycerol, 60g/L);
B= -(30°C), +(37°C)

As can be seen in figure 6.1, cultures at a higher level of temperature grew faster during the first 10 h of the fermentation. This result was expected as specific growth rate is dependant on temperature. *E. coli* cells will grow over a temperature up to 40 °C, and the growth rate increases in response to increasing temperature in a central normal range of its growth temperatures (20 to 37 °C) (Farewell et al, 1998). Nevertheless, it seems that temperature level was not significant to obtain higher biomass levels. Specific growth rate was 0.24 ± 0.01 and 0.15 ± 0.01 for cultures at high and low temperature, respectively.

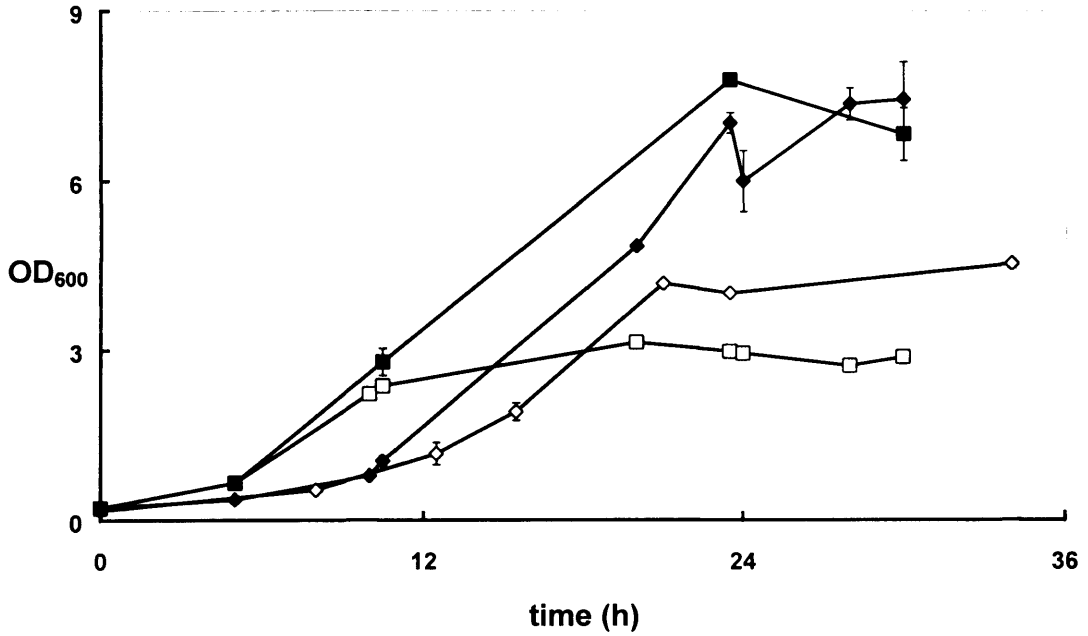


Figure 6.1 Effect of temperature and concentration of nutrients on growth kinetics in fermentations at 250 mL scale (shake flasks): ◇ C-source (normal) & T= 30°C; ▫ C-source (normal) & T= 37°C; ◆ C-source (double) & T= 30°C; ■ C-source (double) & T=37°C. Error bars represent standard deviations of duplicate fermentations.

On the other hand, higher levels of biomass (OD_{600}= 7.5) were reached in cultures with double concentration of nutrients regardless of the temperature. This value was ~1.8 times higher than cultures with normal concentration of glycerol. Based on these results, it could be suggested that cells in this culture could metabolise levels of glycerol up to 60 g/L without growth inhibition. Therefore, doubling the initial concentration of nutrients was the initial strategy to follow at 20 L scale.

Experiments to test the effect of nutrient concentration on growth and product formation in a gas blending system using pulsed fed-batch feeding strategy at 20 L scale are presented in the following section.

6.3.1 EFFECT OF CONCENTRATION OF NUTRIENTS ON FERMENTATION PROCESS PERFORMANCE AT 20 L SCALE

Based on growth kinetics studies in shake flasks, batch-fed fermentations with double concentration of nutrients in a gas blending system at 20 L scale were carried out. As can be seen in figure 6.2, a maximum specific growth rate of ~0.12 h^{-1} was observed in fermentations with double concentration of nutrients. This value was found to be 30% less when compared with batch-fed fermentations with normal nutrient concentrations presented in the previous chapter.

The lower maximum specific growth rate in this type of fermentations led to high levels of glycerol accumulation (up to 75 g/L) when adding glycerol shots as a result of the pulsed fed-batch feeding strategy. Therefore, two approaches were proposed to avoid accumulation of glycerol in the broth: a delay in the glycerol additions time (~4 h based on OD reading) and an extension of fermentation time (12 h). The set of experiments is summarised in table 6.2.

It was hypothesised that higher cell densities could be achieved if levels of glycerol could be maintained at levels below the initial concentration (~60 g/L). Nevertheless, very low yields of Fab' (up to 10 mg/L) were obtained although levels of glycerol did not exceed the initial glycerol concentration. The following sections describe each of these aspects in turn.

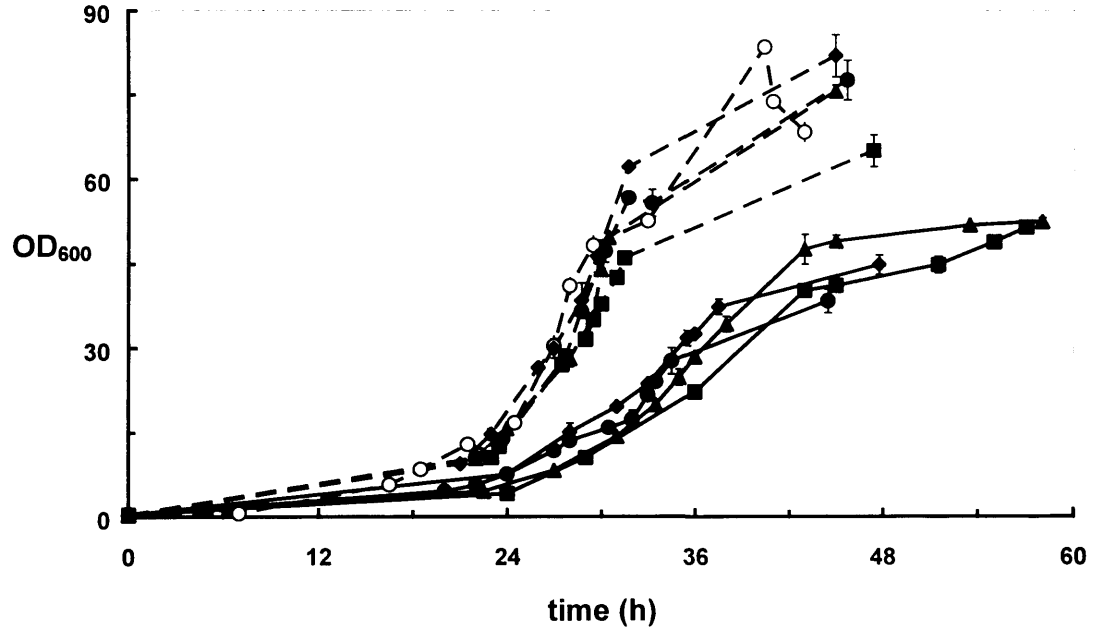


Figure 6.2 Effect of nutrient concentration on growth kinetics in batch-fed fermentations at 20 L scale: a) with normal concentration of nutrients -○- non-GB, -◆- GB-1 (-rpm, -DOT), -◆- GB-2 (+rpm, -DOT), -▲- GB-3 (-rpm, +DOT), -■- GB-4 (+rpm, +DOT), b) with double concentration of nutrients -◆- DS-1 (-OD & - time), -◆- DS-2 (+ OD & - time), -▲- DS-3 (-OD & + time), -■- DS-4 (+ OD & + time). Error bars represent standard deviations of duplicate fermentations.

Table 6.2. Set of experiments to obtain a higher cell density by doubling concentration of nutrients in the culture to produce Fab' at 20 L

Fermentation	A= Additions time	B= Fermentation time
DS-1	-	-
DS-2	+	-
DS-3	-	+
DS-4	+	+

A= -(first shot at OD_{600}= ~15), +(first shot at OD_{600}= ~20);

B= -(48 h), +(60 h)

As can be seen in figure 6.3, biomass levels in fermentations with double concentration of nutrients were ~25 g/L. This value was 15% lower than that in fermentations with normal concentration of nutrients, except for the gas blending fermentation GB-4 (high agitation rate and high DOT level). Where the same level of biomass was obtained.

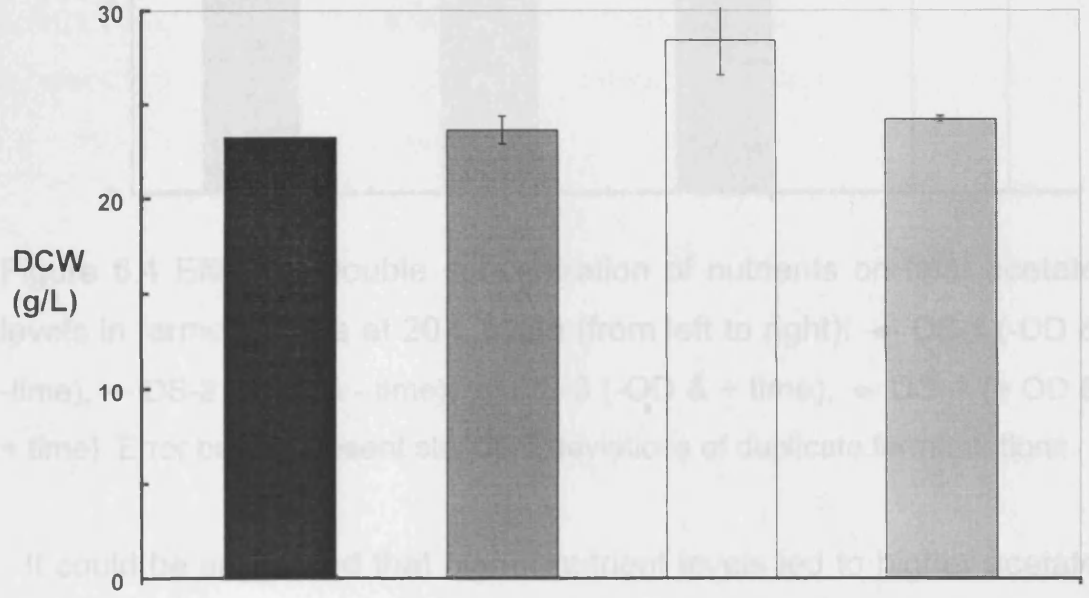


Figure 6.3 Effect of double concentration of nutrients on biomass levels in fermentations at 20 L scale (from left to right): -■- DS-1 (- OD & - time), -■- DS-2 (+OD & - time), -□- DS-3 (- OD & + time), -□- DS-4 (+ OD & + time). Error bars represent standard deviations of duplicate fermentations.

As already discussed, high levels of acetate ~5 g/L were found in fermentation GB-4 and this was suggested as a possible explanation of growth inhibition. Final acetate levels were measured in fermentations with double concentration of nutrients and as can be observed in figure 6.4, levels of 4-7 g/L were measured.

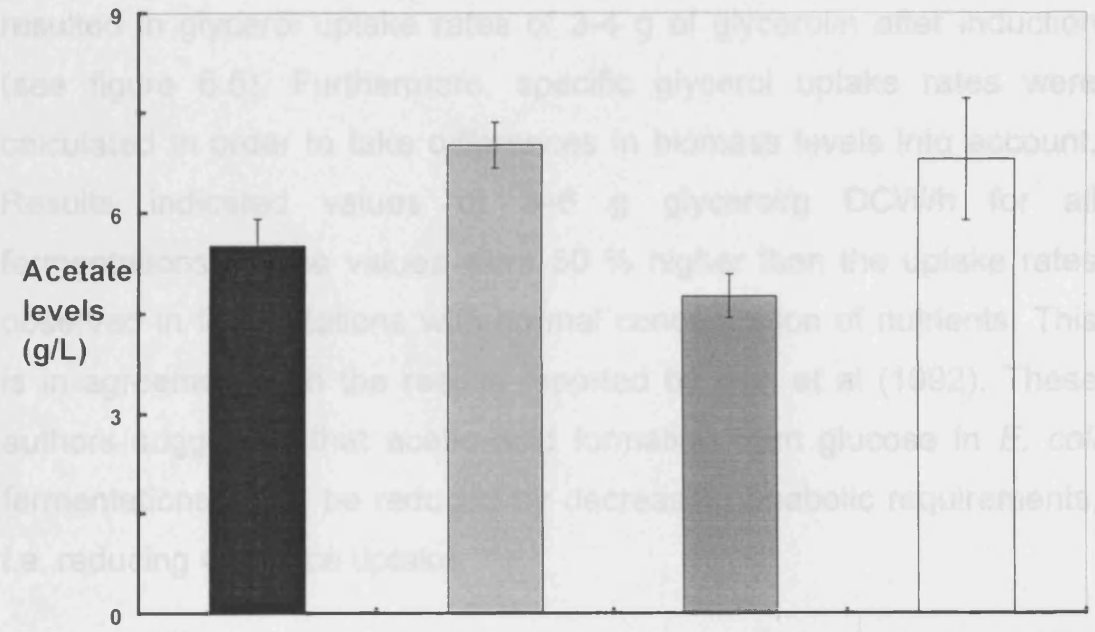


Figure 6.4 Effect of double concentration of nutrients on final acetate levels in fermentations at 20 L scale (from left to right): -■- DS-1 (-OD & -time), -■- DS-2 (+ OD & - time), -□- DS-3 (-OD & + time), -□- DS-4 (+ OD & + time). Error bars represent standard deviations of duplicate fermentations.

It could be suggested that higher nutrient levels led to higher acetate levels inhibiting biomass formation. Acetate is produced both when *E. coli* is grown under oxygen-limiting conditions and/or when carbon flux exceeds the biosynthetic demands and the capacity for energy generation within the cell (Rocha et al, 2002).

The inhibitory effect of high glycerol levels at 20 L scale was not expected based on the results at shake flask scale. Nevertheless, the higher mass transfer in a fermenter than in a shake flask could have led to higher glycerol uptake rates and consequently to higher acetate levels.

In average, all fermentations with double concentration of nutrients resulted in glycerol uptake rates of 3-4 g of glycerol/h after induction (see figure 6.5). Furthermore, specific glycerol uptake rates were calculated in order to take differences in biomass levels into account. Results indicated values of 3–6 g glycerol/g DCW/h for all fermentations. These values were 50 % higher than the uptake rates observed in fermentations with normal concentration of nutrients. This is in agreement with the results reported by Han et al (1992). These authors suggested that acetic acid formation from glucose in *E. coli* fermentations could be reduced by decreasing anabolic requirements, i.e. reducing C-source uptake.

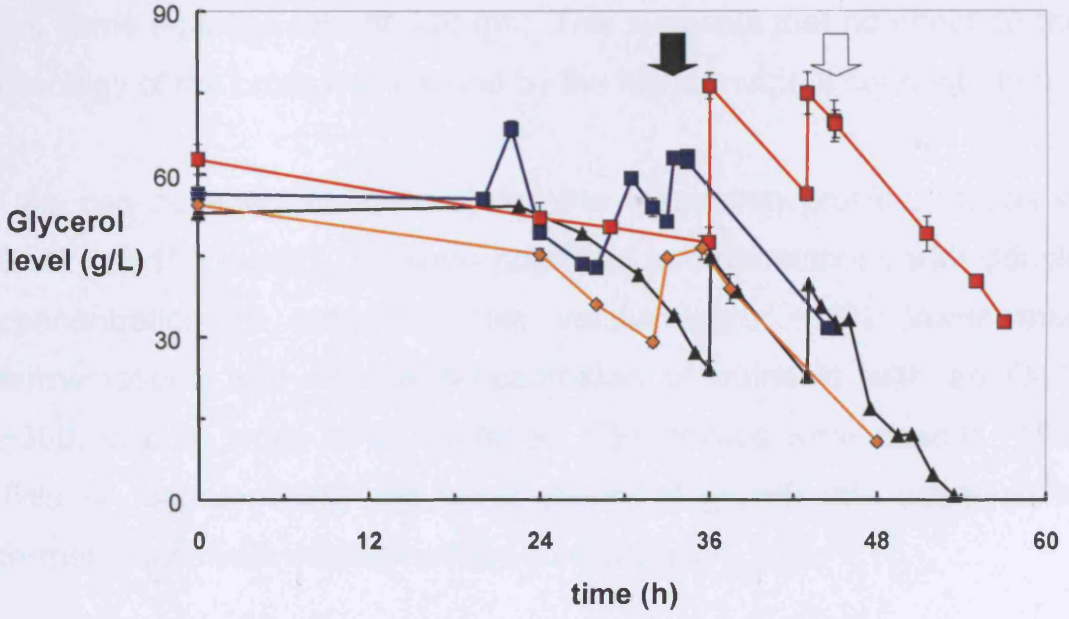


Figure 6.5. Effect of gas blending and nutrient level on glycerol uptake rate of four different fermentations systems at 20 L: DS-1 (-OD & - time), DS-2 (+ OD & - time), DS-3 (-OD & + time), DS-4 (+ OD & + time). Closed arrow indicates first addition of lactose. Open arrow indicates second addition of lactose. Error bars represent standard deviations of duplicate fermentations.

In order to determine the possible reasons for low production in fermentations with double nutrient concentration at 20 L scale and recommend further investigation, the data for the "best case" DS-3 is discussed. Fermentation DS-3 had the lowest levels of acetate (see figure 6.4) and the "highest" levels of Fab' with 7 mg/L (see figure 6.6). All other fermentations observed levels < 5 mg/L of Fab'.

As can be seen in figure 6.6a, a maximum level of 80 % of O_2 in the inlet air flow was required to maintain a constant 30 % DOT level. Furthermore, K_La values of ~400 were observed throughout the fermentation. By comparison, these values were similar to the ones observed in fermentations with normal nutrient concentration and using the same agitation rate of 500 rpm. This suggests that no effect on the rheology of the broth was caused by the higher nutrient concentration.

As can be seen when analysing the respiration profiles, values of OUR of ~100 $mmol.L^{-1}h^{-1}$ were observed in fermentations with double concentration of nutrients. This values were ~60% lower than fermentations with normal concentration of nutrients with an OUR ~300. In both types of fermentation, CER values were around ~100. This is congruent with the lower values of growth rate observed in fermentations with double nutrient concentration.

Levels of ~30 g/L of glycerol were measured at the point of induction (see figure 6.6c). This level of glycerol in the broth was only 40% less than the lactose concentration. Both substrates were consumed in parallel until glycerol depletion. Nevertheless, glycerol was consumed almost twice as fast as lactose.

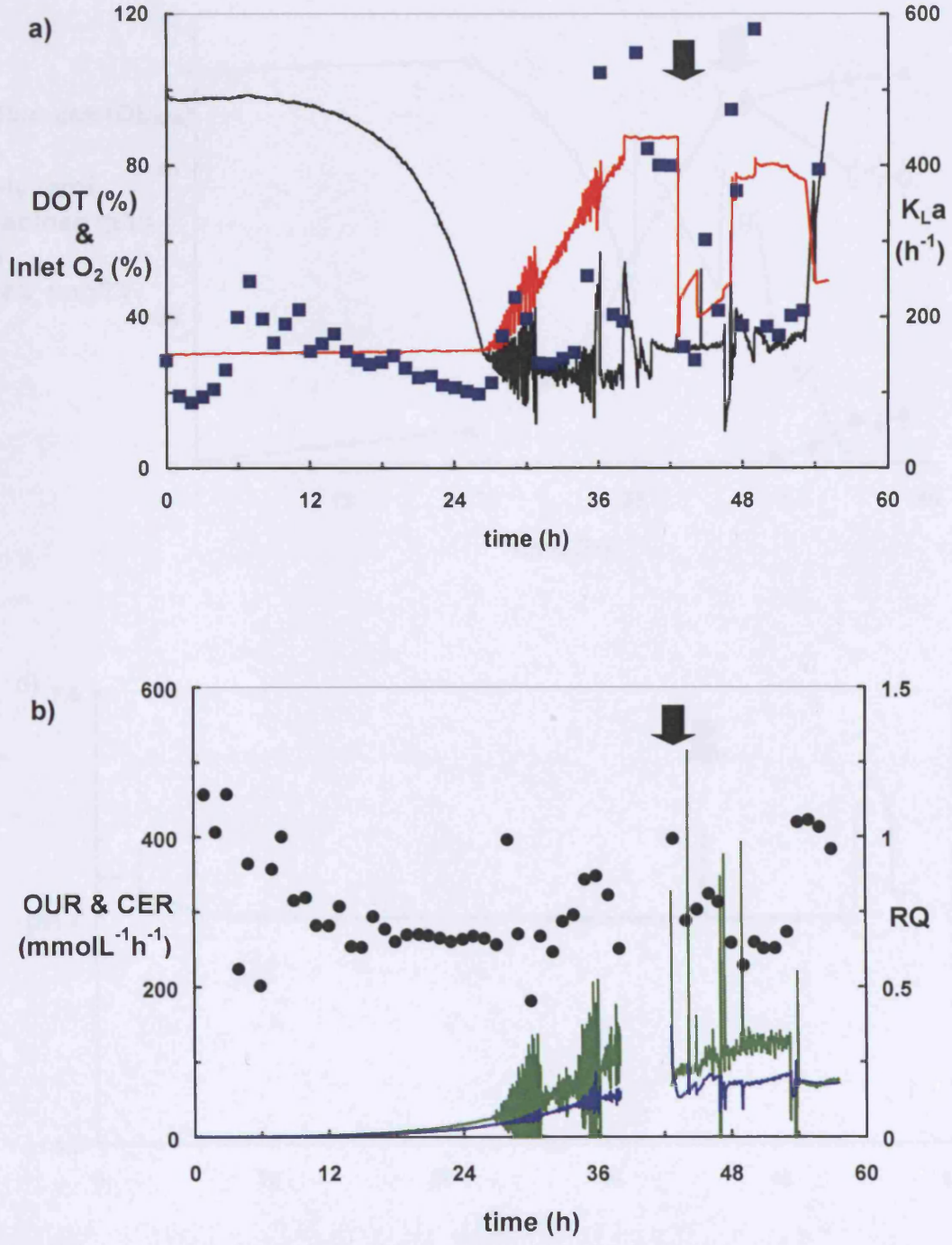


Figure 6.6. Effect of doubling concentration of nutrients in a fermentation (DS-3) at 20 L scale on a) — DOT, — inlet O_2 concentration, -■- K_La, b) — OUR, — CER, and • RQ

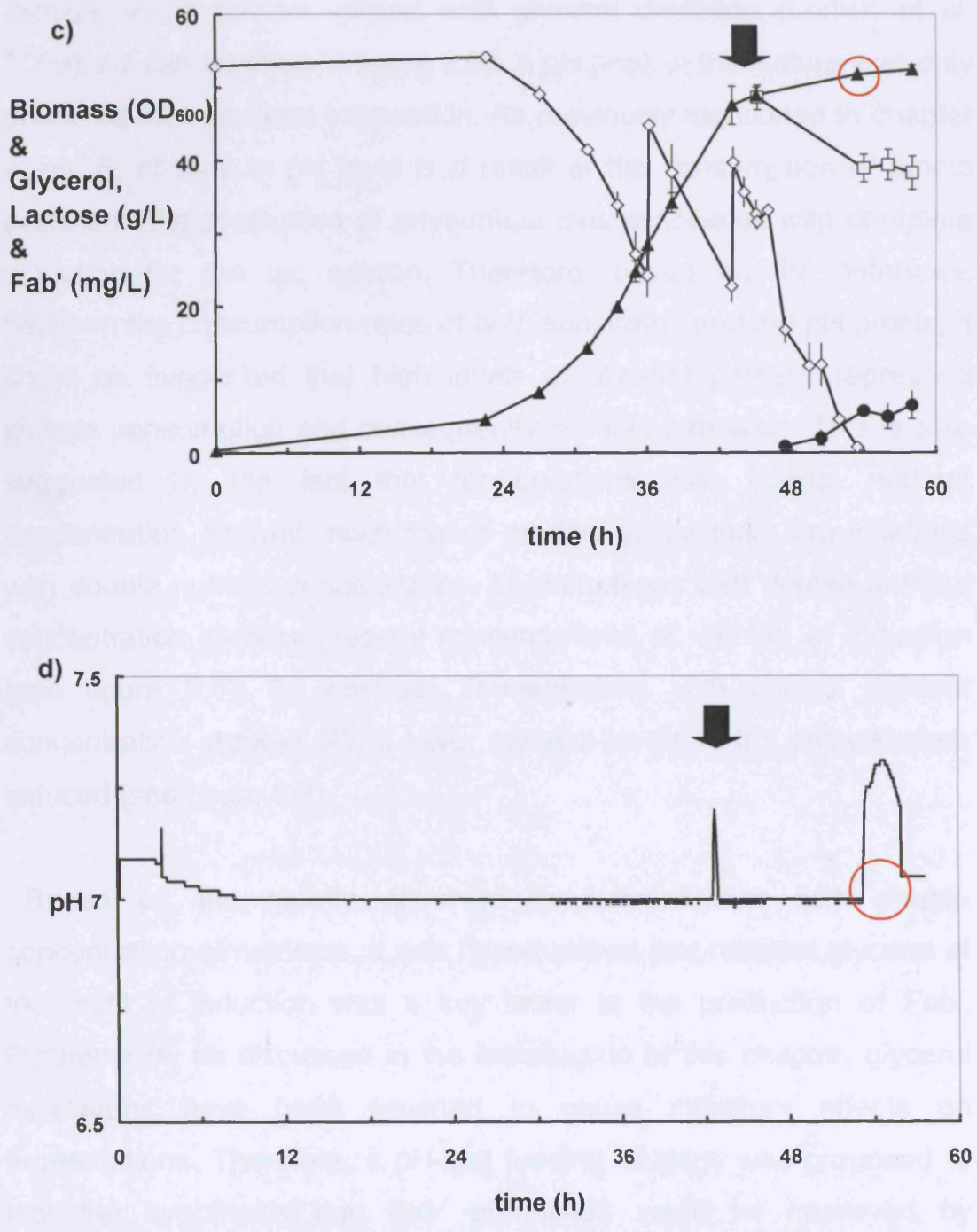


Figure 6.6. Effect of doubling concentration of nutrients in a fermentation (DS-3) at 20 L scale on c) ▲ biomass level, ◇ glycerol level, □ lactose level and ● Fab', d) — pH

As glycerol is utilised by constitutive enzymes and the enzymes for lactose consumption need to be induced, it could be expected that lactose would not be utilised until glycerol depletion (Lodish et al, 2000). As can be seen in figure 6.6d, a pH peak in the culture was only observed after glycerol exhaustion. As previously mentioned in chapter 4 and 5, change in pH level is a result of the consumption of amino acids and the production of ammonium ions associated with complete induction for the lac operon. Therefore, based on the difference between the consumption rates of both substrates and the pH profile, it could be suggested that high levels of glycerol partially repressed lactose consumption and consequently product formation. This is also suggested by the fact that fermentations with normal nutrient concentration showed much higher product yields than fermentations with double nutrient concentration. Fermentations with double nutrient concentration showed glycerol concentrations of ~40 g/L at induction (see figure 6.6). By contrast, fermentations with normal nutrient concentration showed 50 % lower glycerol levels in the culture when induced (see figure 6.7).

Based on the results observed in fermentations with double concentration of nutrients, it was hypothesised that residual glycerol at the point of induction was a key factor in the production of Fab'. Furthermore, as discussed in the introduction of this chapter, glycerol oscillations have been reported to cause inhibitory effects on fermentations. Therefore, a pH-stat feeding strategy was proposed to test the hypothesis that Fab' production could be improved by appropriate control for glycerol oscillations and minimisation of residual glycerol at the point of induction.

6.3.2. Evaluation of pH-stat feeding strategy in the production of Fab' antibody fragments

In order to evaluate the effect of a pH-stat feeding strategy on biomass yields and product levels, a comparison with a repeated batch fermentation was carried out to allow the effects of glycerol oscillations on final Fab' yield, growth kinetics and cellular localisation of the product to be identified. A 2-fold increase in Fab' yield was achieved by implementing a pH-feeding strategy in this process. In table 4 the principal characteristics of the two feeding strategies are summarised. As can be seen, similar amounts of glycerol were added to both processes. Nevertheless, a comparison of the biomass yield on glycerol ($Y_{X/glycerol}$) indicates a 13 % higher glycerol utilisation in the case of pH-stat feeding. A $Y_{X/glycerol}$ of 0.39 and 0.34 was obtained in the pH-feed and repeated batch fermentation, respectively. Furthermore, product yields on biomass ($Y_{product/cells}$) of 5.91 and 3.26 were obtained for the pH-stat feeding and repeated batch fermentation, respectively. Based on this data, it can be concluded that the pH-stat feeding strategy increased both glycerol utilisation and product formation by the cells. Furthermore, when comparing the product yield on lactose, a 50% better lactose utilisation was found using the pH-stat method. The following sections describe each of these aspects in turn.

6.3.2.1. EFFECT OF pH-STAT FEEDING STRATEGY ON GLYCEROL LEVELS

Both fermentations at 20 L scale run in a pulsed fed-batch and pH-stat mode showed similar growth kinetics during the first ~24 h of fermentation (see figure 6.7). Induction with lactose was performed in both fermentations after ~10 hours of growth at maximum specific rate.

Table 6.3. Comparison of repeated batch-feeding vs. pH-feeding strategy in an *E. coli* fermentation to produce Fab' at 20 L scale.

Feeding strategy	Gly. added (g)	Total gly. (g)	DCW (g/L)	Total lactose added (g)	Total product conc. (mg/L)	X yield on gly. $Y_{X/gly.}$ (g/g)	Fab' yield on lac. $Y_{P/S2}$ (mg/g)	Fab' yield on X $Y_{P/X}$ (mg/g)
Repeated batch	750	1125	30.65	1250	100	0.34	1	3.26
pH-feeding	700	1075	33.87	1250	200	0.39	2	5.91

The total working volume in both feeding strategies was 12.5 L

In the pH-stat fermentations a constant level of biomass was achieved in the stationary phase, 48 hours into the process. By contrast, in the pulsed fed-batch fermentation cell lysis was observed after 43 hours of process (see figure 6.7). As for the fermentations which employed a pH-stat no glycerol oscillations were observed throughout the whole process, but rather a continuous exhaustion of the C/energy source. The yield coefficient of biomass on glycerol added was 0.39g of DCW/ g of glycerol in the exponential phase. This result is comparable to the values of 0.4g of DCW/g of glycerol for exponential phase in aerobic fed-batch fermentation with *E. coli* producing a recombinant growth hormone (rhGH) at scales of up to $3m^3$ (Bylund et al, 2000).

The OUR profiles followed similar trends in both cultures until the second lactose addition (see figure 6.8). A stable OUR profile was observed after 48 hrs of fermentation time in the pH-stat culture, while a decrease in OUR was observed in the fed-batch fermentation.

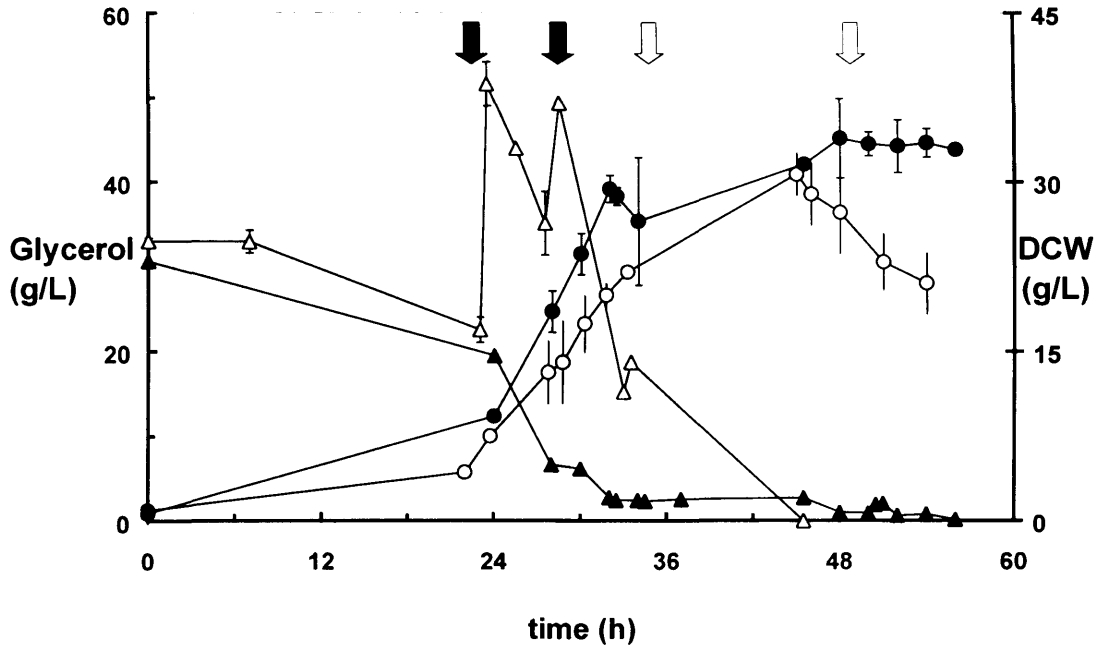


Figure 6.7 Effect of feeding strategy on DCW and glycerol level in 20 L scale Fab' antibody fragment fermentation: -△- Fed-batch (glycerol), -▲- pH-stat (glycerol), -○- Fed-batch (DCW), -●- pH-stat (DCW). The closed arrows represent the additions of glycerol for the fed-batch fermentation and the open arrows represent the additions of lactose for both type of fermentation. Error bars represent standard deviations of duplicate fermentations.

A maximum OUR value of ~200 $mmol.L^{-1}h^{-1}$ was measured in both cultures. Nevertheless, readings were 15 % less when compared with the values of ~300 observed in cultures with the same operating conditions previously reported in section 5.3.1. No further explanation can be given, except that a drift in the mass spectrometer was previously reported by others operators at UCL. As this possible drift effect affected both systems, results were still comparable.

Diauxic growth was observed at ~33 h and ~38 h of fermentation at pH-stat and batch-fed culture, respectively. This ~5 h difference could be explained based on the different glycerol levels at time of induction. Results suggest that in the pH-stat culture, glycerol levels were minimal and therefore the switch to lactose metabolism was complete and faster.

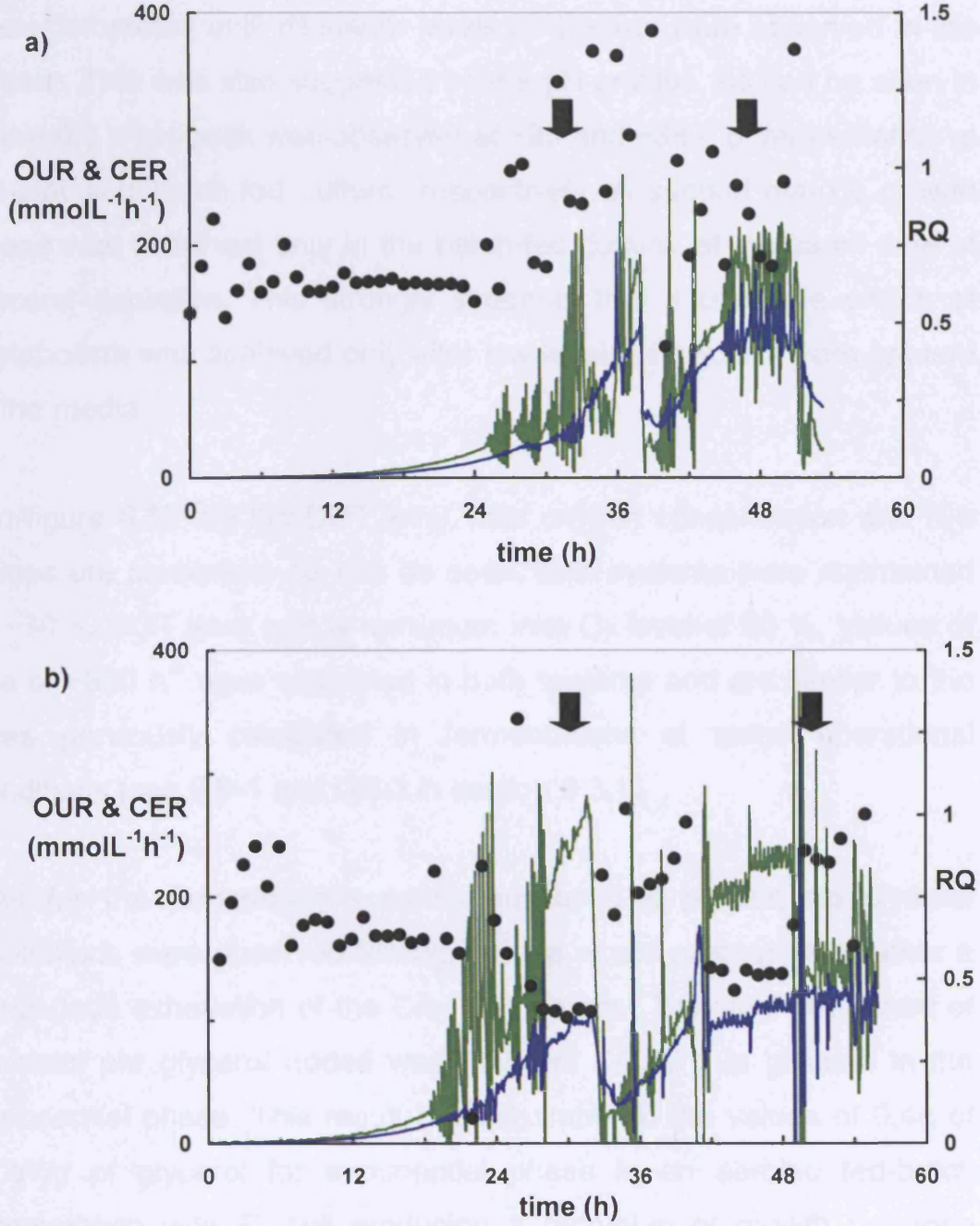


Figure 6.8. Effect of feeding strategy on — OUR, — CER and • RQ on a) batch-fed fermentation and b) pH-stat fermentation, at 20 L scale. Arrows represent addition of lactose.

As for the batch–fed culture, no switch of metabolism seemed to have been completed until minimum levels of glycerol were observed in the culture. This was also suggested by the pH profiles. As can be seen in figure 6.9 a pH peak was observed at ~33 and ~38 h of fermentation at pH-stat and batch-fed culture, respectively. A second diauxic growth phase was observed only in the batch-fed culture, at the same time of glycerol depletion. This strongly suggests that a complete switch of metabolism was achieved only after low levels of glycerol were present in the media.

In figure 6.10 are the DOT level, inlet oxygen concentration and K_La values are presented. As can be seen, both systems were maintained at ~30 % DOT level with a maximum inlet O_2 level of 80 %. Values of K_La of ~300 h^{-1} were estimated in both systems and are similar to the ones previously calculated in fermentations at same operational conditions (see GB-1 and GB-3 in section 5.3.1).

As for the fermentations which employed a pH-stat no glycerol oscillations were observed throughout the whole process, but rather a continuous exhaustion of the C/energy source. The yield coefficient of biomass per glycerol added was 0.35g of DCW/ g of glycerol in the exponential phase. This result is comparable to the values of 0.4g of DCW/g of glycerol for exponential phase in an aerobic fed-batch fermentation with *E. coli* producing a recombinant growth hormone (rhGH) at scales of up to $3m^3$ (Bylund et al, 2000).

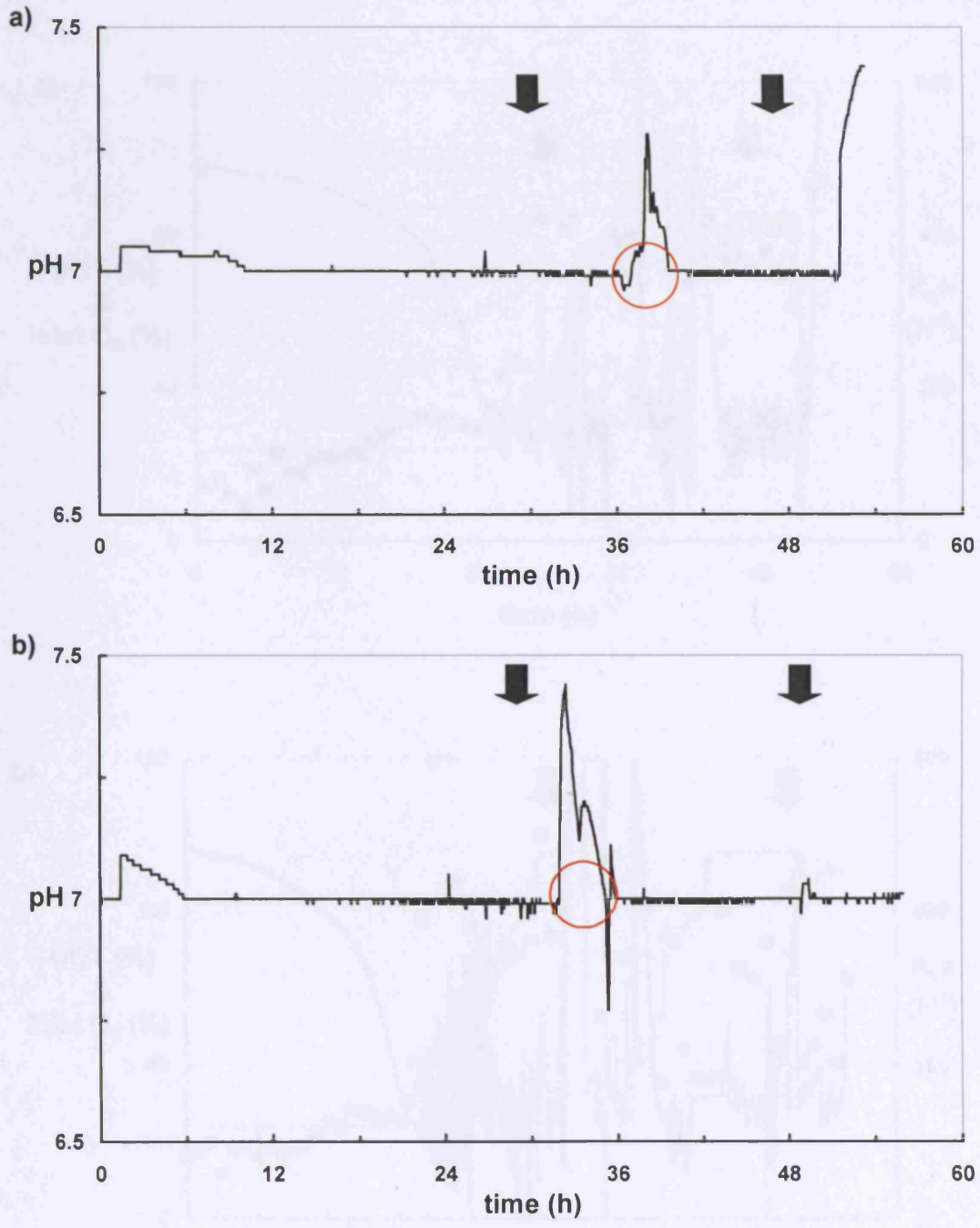


Figure 6.9. Effect of feeding strategy on pH on a) batch-fed fermentation and b) pH-stat fermentation, at 20 L scale. Arrows represent addition of lactose.

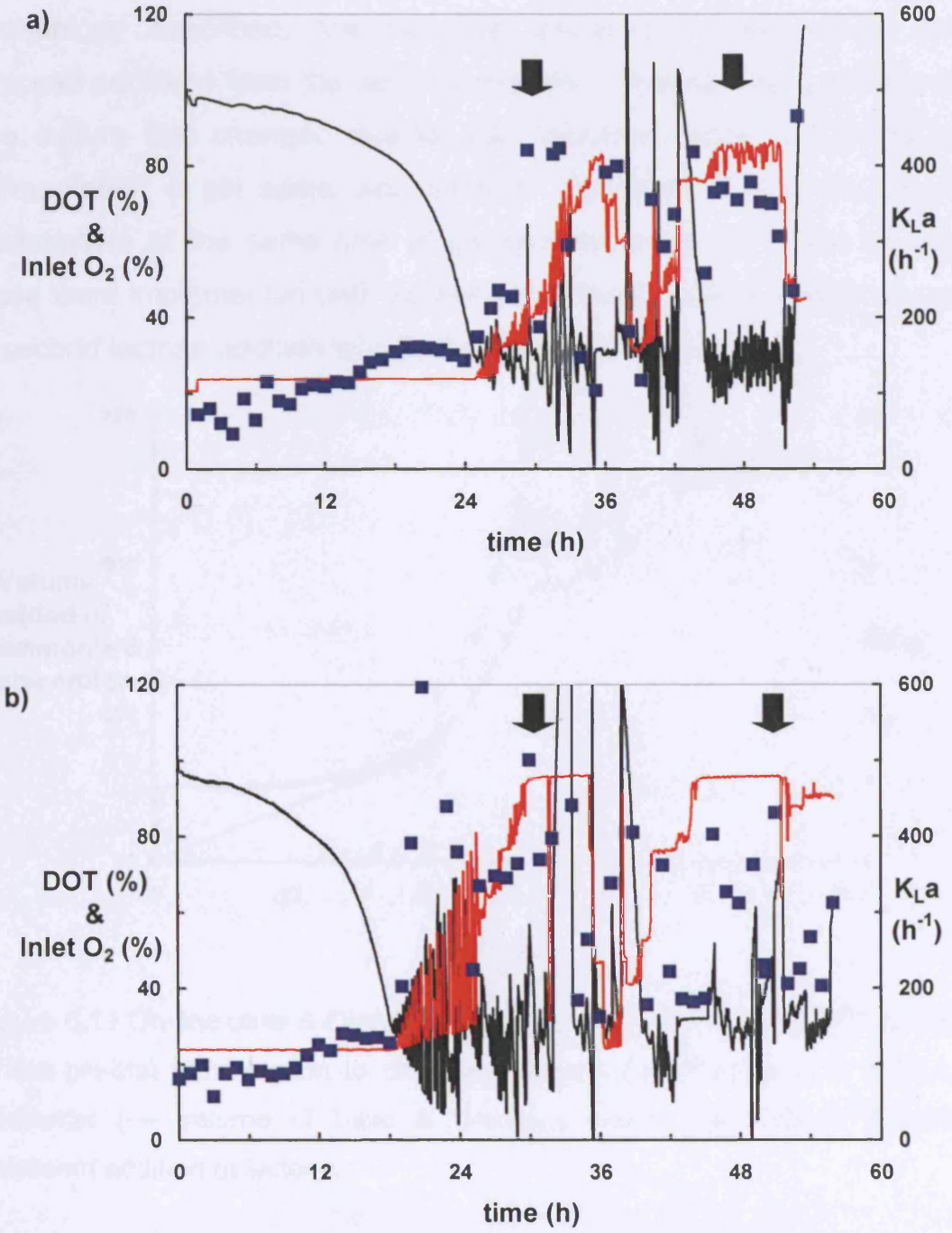


Figure 6.10. Effect of feeding strategy on — DOT, — inlet O_2 concentration, -■- K_La on a) batch-fed fermentation and b) pH-stat fermentation, at 20 L scale. Arrows represent addition of lactose.

As can be seen in figure 6.11, glycerol additions followed an exponential behaviour as expected; paralleling cell growth. In this technology described, the biomass concentration determined the glycerol additions from the amount of alkali to maintain constant pH of the culture that changed due to acid formation. After ~32 hours of fermentation a pH spike was seen in the broth due to switch of metabolism at the same time of glycerol depletion. Additions of only base were implemented until exhaustion of the C/source energy where a second lactose addition was performed.

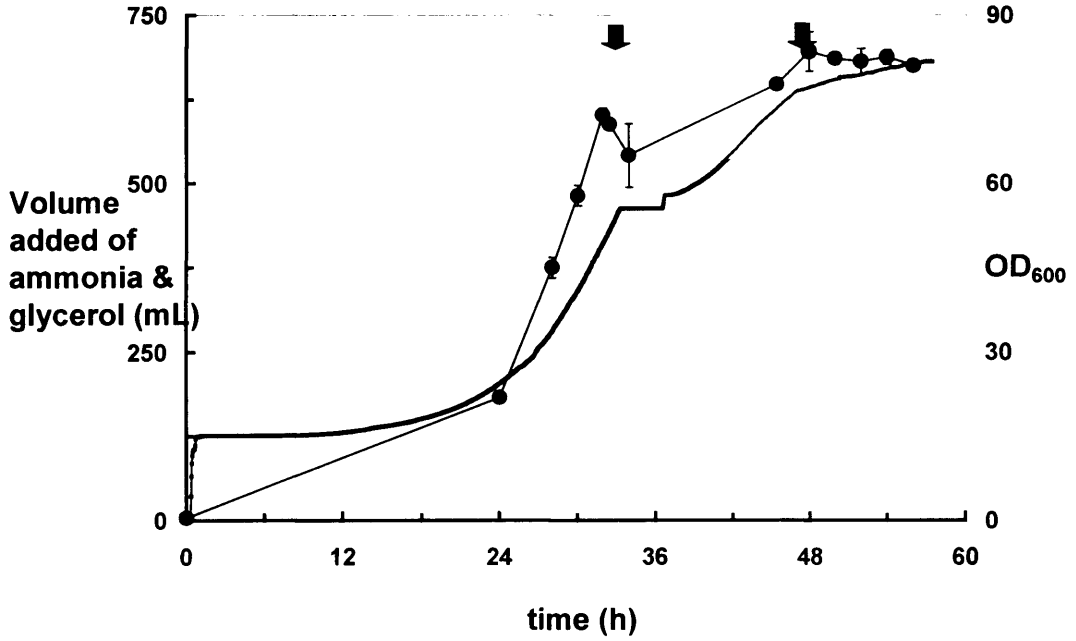


Figure 6.11 On-line base & C/energy source profile and off-line OD600 profile of the pH-stat fermentation to produce Fab' antibody fragments in a 20 L fermenter (— volume of base & C/energy source, -◆- OD_{600}). Arrows represent addition of lactose.

The way of glycerol additions proposed could be considered as a variation of a pH-auxostat. In this technique, the change of environmental parameters leads only to a relatively small effect on biomass concentration at near optimal growth conditions (Kasemets et al, 2003).

A linear correlation between glycerol additions and biomass accumulation was obtained for the exponential phase (see equation 1). The biomass of the culture followed a typical exponential growth (see equation 2):

$$V(t)_{glycerol} = 10.3X(t) - 22.6 \qquad \text{eq. (1)}$$

$$X(t) = X_0 e^{\mu t} \qquad \text{eq. (2)}$$

where $V_{glycerol}$ = volume of glycerol additions (mL), X(t) = biomass accumulation (g/L), X_o= initial biomass concentration (g/L), μ = maximum specific growth rate (h^{-1}), t = time (h)

Combining equation 1 and 2

$$V(t)_{glycerol} = 10.3X_0 e^{\mu t} - 22.6 \qquad \text{eq. (3)}$$

Therefore, maximum specific growth rate could be controlled in this fermentation process using the additions of glycerol and initial biomass, as follows:

$$\mu = LN\left(\frac{V(t)_{glycerol} + 17.8}{4.1X_0}\right)\frac{1}{t} \qquad \text{eq. (4)}$$

This correlation is only valid for this particular fermentation. Further work is needed to validate this correlation under other operational conditions.

6.3.2.2 EFFECT OF pH-STAT FEEDING STRATEGY ON BIOMASS LEVELS

A maximum specific growth rate of 0.14 h^{-1} and 0.15 h^{-1} in exponential phase was obtained for the repeated batch and pH-stat fermentations, respectively. The low specific growth rates are likely to be the result of the high initial glycerol concentration limiting growth in this defined medium. As illustrated in figure 6.7, for the pH-stat fermentation the biomass level obtained at the time of induction was 26% higher than that for the repeated batch fermentation. The biomass level remained almost constant after induction using the pH-stat strategy, whereas the concentration increased by ca. 30% in the repeated batch process to reach a similar level to that in the pH-stat after 45 hours (~9 hours post induction). However, shortly after this point the significant decrease in biomass concentration was observed in the repeated batch culture in parallel with the exhaustion of glycerol, the main C/energy source. By contrast the cell concentration in the pH stat fermentation remained constant throughout the process. In this system, lactose acts as inducer of product formation and therefore a reduction of growth is expected due to the switch of metabolism to product expression leading to a drain in C/energy source. After induction, cell growth and metabolic activity are often influenced by the on-going expression of recombinant proteins within the cell (Andersson et al, 1996). Induction of recombinant protein synthesis in high-cell density cultures can impose an additional metabolic burden on the host cell, thereby inducing a variety of different stress responses (Andresson et al, 1996b), Neubauer et al, 2003). In some cases, this may even result in increased cell lysis, as has been indicated by the accumulation of DNA and proteins outside the cells (Bylund et al, 2000, Shokri et al, 2003).

It is noticeable that there is a significant difference in the residual glycerol concentration (20 and 2.5 g/L) at the point of induction for the repeated batch and pH-stat cultures respectively (see figure 6.7). This difference could have affected the fermentation performance with regard to acetate formation and lactose utilisation and thus could have caused an additional strain on the cells. It has been reported that the accumulation of the main C-source in the fermentation medium promotes the growth of plasmid-free cells due to possible competition for cellular resources used for the synthesis of the recombinant protein product (β-glalactosidase) resulting in a lower synthesis of cellular house-keeping proteins (Lee et al, 1987). This additional energy demand known as "metabolic burden" could be a possible explanation for the results obtained with regard to biomass accumulation observed in the batch-fed process.

Acetate levels in both fermentations did not exceed 3 g/L and because this value is lower than the reported growth-inhibitory acetate concentration of > 5 g/L (Lee, 1996) reasons other than acetate accumulation are probably responsible for the cessation of cell growth and protein synthesis. As mentioned in the previous chapter, the excretion of acetate under fully aerobic conditions on glycerol has been reported by other authors (Korz et al, 1996 and Macaloney et al, 1997)

As shown in figures 6.12 and 6.13, there are two cycles of acetate production and re-consumption; at ca. 30 h and 45 h of the fermentations. In the repeated batch culture, both cycles reached maximum values of ~2-2.5 g/L whereas for the pH-stat culture significantly lower acetate levels of < 0.5 g/L and ca. 1.5 g/L for the first and the second cycle, respectively.

It has been reported that acetate is produced when carbon flux exceeds the biosynthetic demands and the capacity for energy generation within the *E. coli* cell (Lee, 1996). Although this explanation is largely based on experiments whereby glucose was used as C/energy source it is feasible that a similar imbalance between substrate uptake and the demands for anabolism exist for growth on glycerol. This could be a possible explanation for the first acetate cycle in the repeated batch culture where the levels of glycerol were above 20 g/L before induction. The second cycle in the repeated batch culture seems to be related to lactose consumption and product formation since acetate concentration peaked at the same time as the Fab' yield. By contrast, acetate formation only appeared during the transition period between the lactose depletion and the second lactose addition, but not during product formation in the pH-stat culture.

6.3.2.3. EFFECT OF pH-STAT FEEDING STRATEGY ON Fab' PRODUCTION

Both fermentations reached 100 mg/L of Fab' after 48 hours of cultivation time. However, in the case of the pH-stat a significant increase in product titres after a second lactose addition was obtained whereas no further increase was found for the repeated batch process. In both fermentations the level of periplasmic product localisation was high, with > 90% being retained in the periplasm (see figures 6.12 & 6.13).In the repeated batch fermentation, results showed a rate of 0.94 mg of Fab' produced/g of lactose consumed for the first induction period with a production rate of 9.4 mg Fab'/h. Fab' production after the 2nd induction decreased significantly. By contrast in the pH-stat fermentation, results showed two different rates of 0.42 and 1.23 mg of Fab' produced/ g of lactose consumed for the first and second induction period, respectively (see figure 6.12 & 6.13). In addition, the rate of Fab' production doubled from ~6 mg Fab'/h to ~12 mg Fab'/h from the first to the second period of induction.

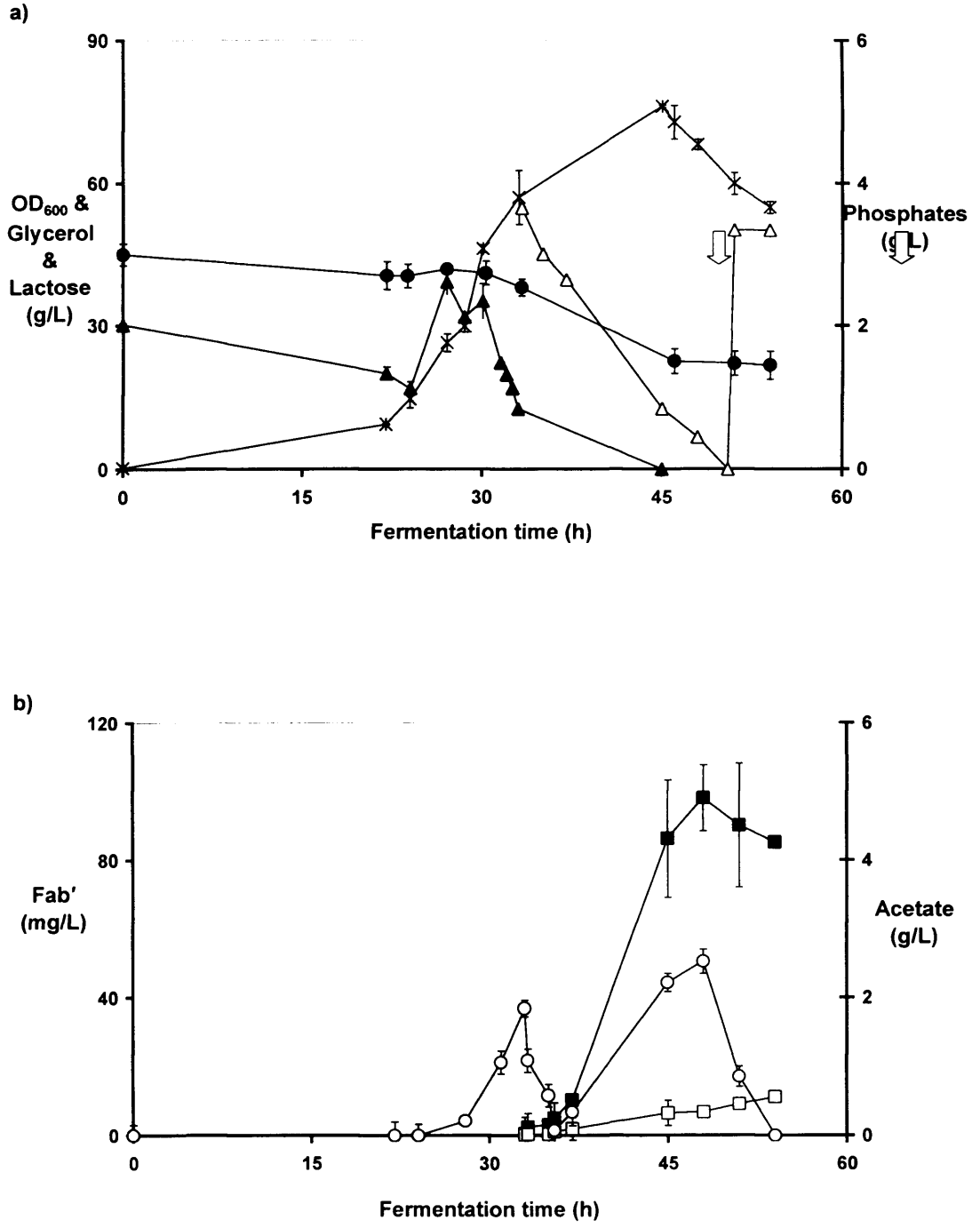


Figure 6.12 Effect of feeding strategy on a) ✳ biomass level (OD_{600}), substrates concentration (● phosphates, ▲ glycerol and △ lactose) and b) by-product concentration (○ acetate) and product formation in periplasm (■ intracellular Fab') and supernatant (□ leakage), in batch-fed fermentation at 20 L scale. Error bars represent standard deviations of duplicate fermentations.

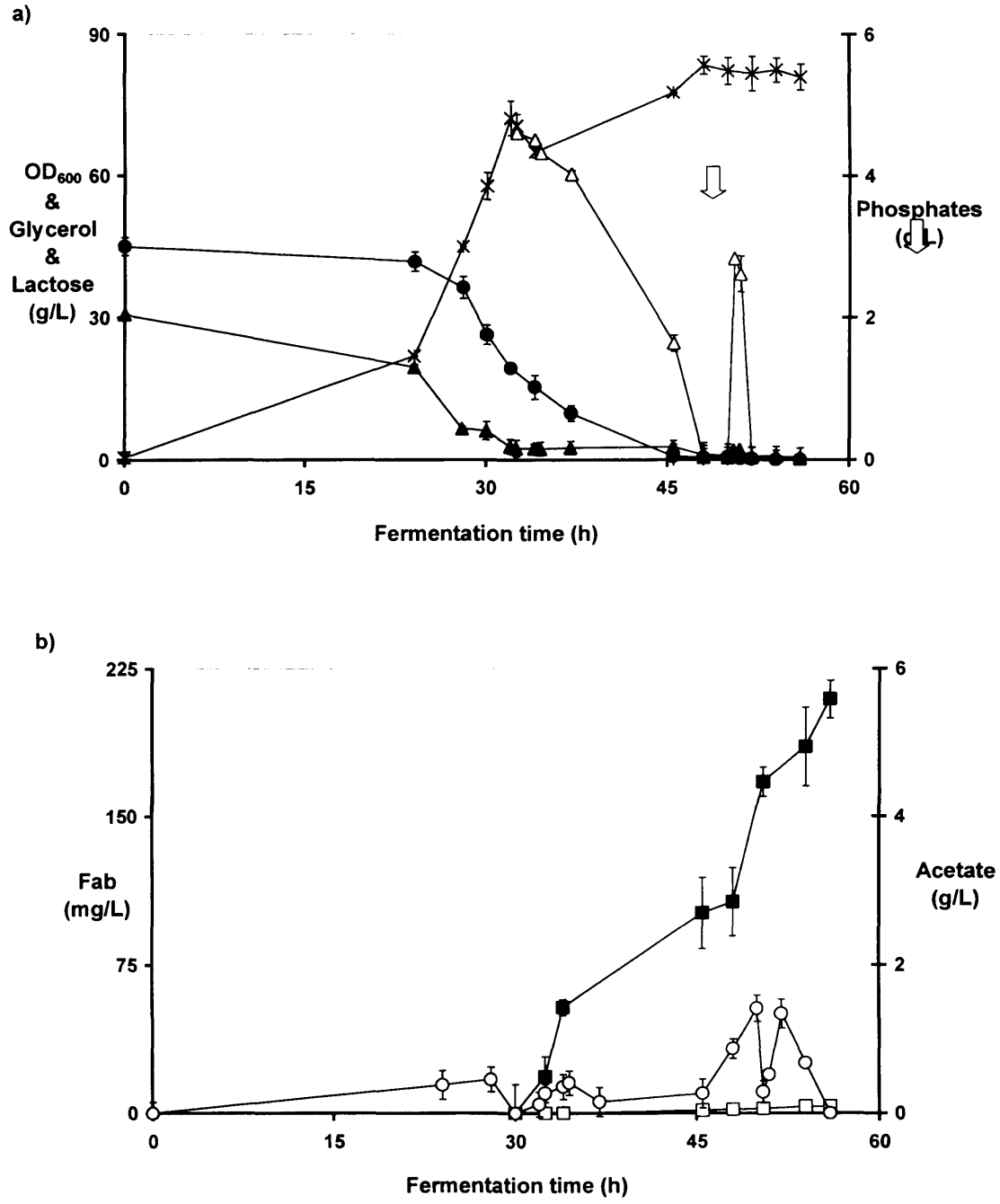


Figure 6.13 Effect of feeding strategy on a) ✳ biomass level (OD_{600}), substrates concentration (-●- phosphates, -▲- glycerol and -△- lactose) and b) by-product concentration (-○- acetate) and product formation in periplasm (-■- intracellular Fab') and supernatant (-□- leakage), in pH-stat fermentation, at 20 L scale. Error bars represent standard deviations of duplicate fermentations.

The results found in the pH-stat fermentation might be due to the fact that a pseudo-stationary phase with constant cell concentration was reached by the time of the second lactose addition and therefore more C/energy source were available for product formation as opposed to biomass synthesis. The onset of this phase is likely to be the result of phosphate limitation at the time of the second lactose addition. At the time of the first lactose addition, phosphate levels of 2.54 and 1.28 g/L were determined for the repeated batch and pH-stat fermentations, respectively (see figure 6.12 & 6.13). After ~13 hours of induction (before the second addition of lactose) the pH-stat culture showed a complete depletion of phosphate whereas for the repeated batch culture a final level of 1.45 g/L was observed.

It is also noticeable that no significant phosphate consumption was observed until the last addition of glycerol in the repeated batch fermentation. By contrast, the pH-stat culture consumed phosphate continuously until depletion. The lack of utilisation in the repeated batch culture could be the result of glycerol oscillations that could lead to additional stress conditions. On the other hand the levels of phosphates did not affect the productivity during the first period of induction although it has previously been reported that increasing phosphate concentration at large scale resulted in increased release of periplasmic material into the extracellular broth (Bowering, 2000).

6.3.2.4. IMPACT OF FEEDING STRATEGY ON CENTRIFUGAL RECOVERY OF Fab'

The effect of the feeding strategy on initial DSP recovery was determined by evaluating product release occurring in the centrifugation step (see figure 6.14). Oscillations of C/energy source have been reported to cause negative physiological responses during the production phase that could lead to cell lysis during a stress situation (Lin et al, 2000).

The level of periplasmic Fab' (product) and intracellular protein (contaminant) release associated with the continuous disk stack centrifugal recovery of cells obtained from fermentations operated under the two feeding strategies was investigated. In both cases 98% clarification was achieved and a difference in product loss of less than 1% was observed between fermentations (see figure 6.14). Therefore, the pH-stat strategy contributed to improving Fab' productivity without compromising primary recovery of cells by maintaining constant low levels of glycerol.

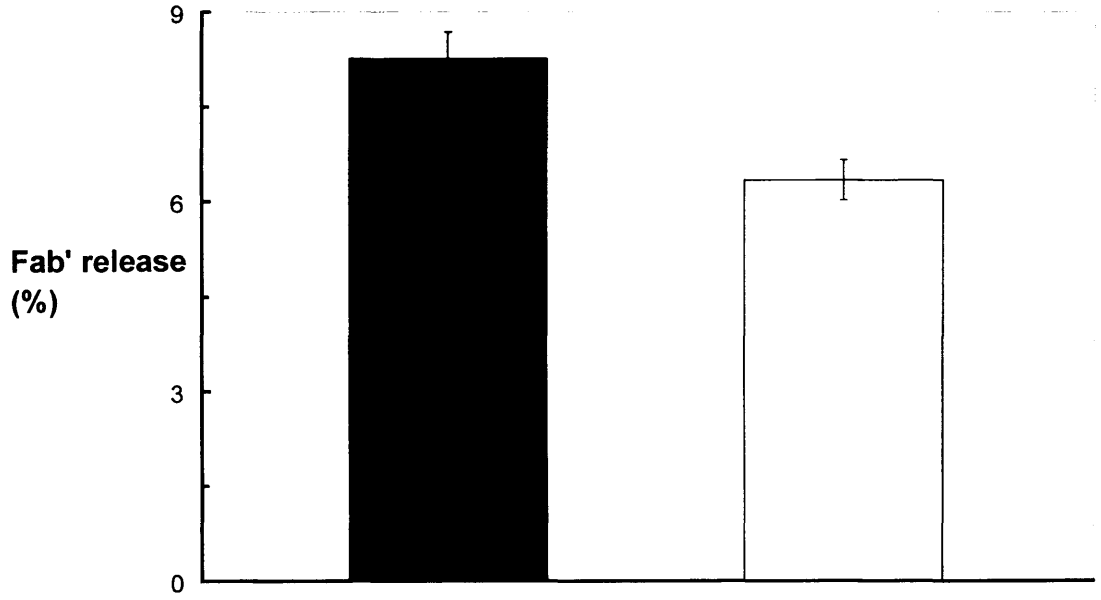


Figure 6.14. Effect of feeding strategy on Fab' release in the centrifugation step in -■- batch-fed and -□- pH-stat fermentation at 20 L scale. Error bars represent standard deviations of duplicate fermentations.

In this chapter a comparison between Fab' produced under batch-fed and pH stat feeding strategies has been presented. The hypothesis that increased Fab' titres can be achieved in a fermenter system that has no glycerol oscillations (pH-stat) has been tested. The effect of feeding strategy on growth kinetics, Fab' yield, and cellular localisation, has been determined. Results suggest that a pH-stat feeding strategy was more effective for increasing Fab' production than a batch-fed feeding strategy.

The results reported in this work suggest a fermentation feeding strategy that takes advantage of the pH-control available in bioreactor systems such that base additions are made in parallel with increasing biomass levels.

A pH-stat feeding strategy was successful in avoiding glycerol oscillations. At 20 L scale a 2-fold increase in the production of Fab' via an extended induction phase, compared to a fermentation operated in a pulsed fed-batch mode was achieved. A consistently high level (>90%) of product localisation in the periplasm was also obtained. The results presented here suggest an alternative feeding strategy to increase Fab' yields and further work is needed to evaluate the possibility of scaling up this process.

Chapter 7

Economic Considerations

7.1. ABSTRACT

A preliminary economic analysis of the fermentations carried out at 20 L scale with and without gas blending is presented. The analysis was based on comparing fermentation production costs in £/mg using three different fermentation strategies: batch-fed (non gas blending), batch-fed (gas blending), and pH-stat (gas blending). Results suggested significant cost savings of up to 75% on production costs when implementing pH-stat feeding strategy and gas blending together. This provides an impetus to continue research to evaluate the potential of gas blending and pH-stat feeding strategy in the industrial production of Fab′ antibody fragments.

7.2. INTRODUCTION

As already discussed, there are many different applications of Fab′ fragments in the industry sector, such as: pharmaceutical, food, cosmetic and toiletries, detergents, manufacturing, environmental and biosensors. Current prices for Fab' fragments are between 90 to 190 £/ mg (AMS Biotechnology Ltd., Abingdon, UK and Abcam Ltd., Cambridge, UK) for research use only and not intended for diagnostic or therapeutic use.

Nevertheless, these prices cannot be directly compared to the fermentation cost as it is known that downstream process can account for up to 60% of the total cost of a process (Lienqueo et al, 2000). Consequently, the present economic analysis was focused on evaluating effects of gas blending and feeding strategy at 20 L scale on fermentation product cost.

Any engineering project *per sé* should include an economic analysis, in either absolute or relative terms. The economic analysis presented here was based on work developed at Instituto Politécnico Nacional (Mexico City, Mexico) to evaluate project investments in chemical industries (García-Arrazola et al, 2000).

According to the American Association of Chemical Engineers (AACE) five levels of precision exist to estimate the cost of processes (Garret, 1989): 1) order of magnitude or very preliminary, 2) preliminary based on factors, 3) preliminary, 4) definitive, 5) detailed. The three last studies are usually done by consultancy firms and are performed when asking for financial support for specific projects. The preliminary study based on factors uses correlations relating to the principal equipment cost and commonly represents an error of 30 to 50%.

The production of an antibody fragment from an *E. coli* fermentation has being used to provide a case study for an economic comparison between conventional and disposable-based technology (Novais et al, 2001). Costing models indicated that the capital investment required for a disposable-based technology is 60% less than a conventional option, but running costs are 70% higher. To asses the profitability of each option, the net present value (NPV) was calculated using a standard discounted cash flow technique. The NPV analysis indicated that a disposable-based plant could be economically and conceptually attractive, assuming shorter implementation times and faster time to market.

Decisional support tools have also been applied to investigate process strategies early in the development of a candidate drug. Evaluation of the process from both technical and business perspectives is often too risky to conduct in a full-scale facility. Therefore, the modelling of bioprocesses could be very valuable alternative for evaluating appropriate allocation of resources and accelerate process development.

The aim of this study was to assess the economic feasibility of the fermentations strategies with gas blending proposed in this project. This was done by comparison of final product costs (£/mg) in three different fermentation strategies: batch-fed without gas blending, batch-fed with gas blending and pH-stat with gas blending.

Estimation of product cost was performed using empirical factors according to Peters et al (1990), assuming an imaginary setup of a plant for each process (see section 3.5). Values such as fixed capital, and operating costs were calculated from expenses of the main equipment, raw materials and services on an annual basis. The estimation is for a 20 L scale process, as a first approach. It is hoped that this study could provide initial and valuable information towards manufacturing of Fab' fragments in a gas blending system using a pH-stat feeding strategy at large scale.

7.3 RESULTS AND DISCUSSION

7.3.1. Effect of gas blending and feeding strategy on total capital investment

In table 7.1 the effect of gas blending and feeding strategy on the total capital investment is presented. All calculations were based on data of process equipment cost.

Other costs such as: total cost of the plant (including direct and indirect costs), costs that are not of the plant, fixed capital, and working capital investment were estimated based on equipment cost. Sum of all this costs was defined as the total capital investment. As can be appreciated, the cost of equipment required differs by less than 10% between the different fermentation strategies; therefore, total capital investment differs within the same range. Based on empirical factors, a total of £350,000-381,500 would be required to start the production of Fab' at 20 L scale.

Estimation of expenses per fermentation was performed based on the unit cost and concentration used per fermentation run which has already been shown in section 3.5. Results are summarised in table 7.2. Raw materials (complex media and defined media), additives (antibiotic, cation solutions, etc), inducer (lactose), gas blending, and electricity were considered in this aspect.

7.3.2. Effect of gas blending and feeding strategy on fermentation costs

As can be seen in table 7.2, pH-stat fermentations in a gas blending system resulted in the most expensive process due to the additional cost of oxygen supply and lactose. Results suggest that the difference between the costs of the three processes is no more than 15 %. As can also be observed, electricity does not seem to be significant in the process cost. Therefore, the 12 h additional fermentation time in the pH-stat process seem to have no significant economic implications at this scale. Nevertheless, higher electricity demands might be expected at large scale and might be an important issue to be taken into account. A major issue regarding the supply of O_2 at large scale and the costs associated with this will have also to be considered for more detailed studies.

Table 7.1 Effect of gas blending and feeding strategy on the total capital to invest in a Fab' fermentation process

Component	Cost (£)		
	Fed-batch (NGB)	**Fed-batch (GB)**	**pH-stat (GB)**
EQUIPMENT OF THE PROCESS[1]	**55,000**	**60,000**	**60,300**
National	55,000	60,000	60,300
Importation	-	-	-
a. Delivery, insurance and taxes	2,200	2,400	2,412
- National equipment process	2,200	2,400	2,412
- Importation equipment process	-	-	-
b. Installation of the equipment process	24,856	27,115	27,251
c. Instrumentation and control (installed)	19,250	21,000	21,105
d. Tubing (installed)	34,904	38,077	38,267
e. Equipment and electrical material	5,817	6,346	6,378
f. Services (installed)	37,019	40,385	40,587
g. Buildings (including services)	9,519	10,385	10,437
h. Preparation of yard	5,288	5,769	5,798
i. Soil	3,173	3,462	3,479
DIRECT TOTAL COST OF PLANT	**199,227**	**217,338**	**218,425**
j. Engineering and supervision	19,126	20,864	20,969
k. Costs of construction	23,708	25,863	25,993
INDIRECT TOTAL COST OF PLANT	**42,834**	**46,728**	**46,961**
TOTAL COST OF THE PLANT	**242,061**	**264,066**	**265,387**
l. Honoraries	12,103	13,203	13,269
m. Unexpected	24,206	26,407	26,539
n. Other costs[2]	36,309	39,610	39,808
TOTAL COSTS THAT ARE NOT OF TO THE PLANT	**72,618**	**79,220**	**79,616**

[1] without delivery from the suppliers; [2] previous costs and starting of the plant were considered to be 5 and 10% of the total cost of the plant, respectively.

Table 7.1 (continuation) Effect of gas blending and feeding strategy on the total capital to invest in a Fab' fermentation process

Component	Cost (£)		
	Fed-batch (NGB)	Fed-batch (GB)	pH-stat (GB)
FIXED CAPITAL INVESTMENT	314,679	343,286	345,003
WORKING CAPITAL INVESTMENT[3]	33,319	36,348	36,530
TOTAL CAPITAL INVESTMENT	**347,998**	**379,634**	**381,532**

[3] considering starting operation conditions of 60 % of the design capacity

It was considered that fermentation time in the pH-stat fermentation does not imply a serious delay in the whole manufacturing process (including downstream process). The same number 55 runs per year for both systems with gas blending was assumed as a first estimation. More detailed studies might consider fermentation time difference in estimating the total number of runs per year for the pH-stat strategy.

Cost of raw materials (complex and defined media), additives (antibiotic, cations solutions, etc), inducer (lactose), gas blending, and electricity were used to estimate fermentation product cost. In table 7.3 the effect of gas blending and feeding strategy on final cost of Fab' in £/mg is summarised.

7.3.3. Effect of gas blending and feeding strategy on fermentation product costs

As can be seen in table 7.3 other costs such as: direct materials and services (including raw materials, additives and services), direct labour, fixed costs, and general costs, were estimated. The sum of all these costs was defined as the operating costs. The sum of operating cost, R & D cost and unexpected costs represented the total fermentation production cost per year.

Table 7.2 Effect of gas blending and feeding strategy in the cost per run of a fermentation process to produce Fab'

Material	Cost (£/run)		
	Fed-batch (non-GB)	Fed-batch (GB)	pH-stat (GB)
Complex media (inoculum)	2	2	2
Defined media[1]	12.5	12.5	12.5
Additives	30	30	30
Lactose[2]	4.5	4.5	9
Gas blending[3]	0	2	4
Electricity[4]	0.4	0.04	0.05
Total	**49.4**	**53.04**	**57.55**

[1] working volume of 12.5 L including inoculum

2 considering two additions of lactose in the pH-stat fermentation

3 considering use of ½ and 1 oxygen cylinder in the batch-fed and pH-stat gas blending fermentations

4 considering fermentation time of 48 h and 60 h in the batch-fed and pH-stat fermentations, respectively.

As previously discussed in chapter 5 and chapter 6, gas blending and pH-stat feeding strategy led to higher total Fab' yields compared to that of a traditional fermentation strategy. As can be seen in table 7.3, by comparison with batch-fed fermentation without gas blending, a 4.5-fold and 7-fold higher Fab' yield was observed in batch-fed and pH-stat fermentations with gas blending, respectively. When comparing batch-fed (non-gas blending) with pH-stat (gas blending) fermentation, cost savings of up to 78 % were estimated.

Inclusion of DSP studies is necessary in order to have a complete picture of the process and estimate a final price of Fab' to compare with current market prices. Final application of the product has to be considered as well in the purification stages before comparing currently production costs for a final market price.

Table 7.3 Effect of gas blending and feeding strategy on the final production cost of a fermentation process to produce Fab'

Component	Fed-batch (non-GB)	Fed-batch (GB)	pH-stat (GB)
I. DIRECT MATERIALS AND SERVICES	**24,537.85**	**26,711.94**	**27,078.42**
Raw materials[1]	797.50	797.50	797.50
Additives[1]	1,650.00	1,650.00	1,650.00
Inducer (lactose)[1]	247.50	247.50	495.00
Oxygen supply[1]	0.00	220.00	220.00
Electricity[1]	22.00	2.20	2.75
Maintenance	18,880.74	20,597.17	20,700.15
Supplement of operation	2,832.11	3,089.57	3,105.02
Laboratory expenses	108.00	108.00	108.00
II. DIRECT LABOUR	30,360.00	30,360.00	37,950.00
Operation	26,400.00	26,400.00	33,000.00
Supervision	3,960.00	3,960.00	4,950.00
DIRECT COST OF OPERATION	**58,749.85**	**60,923.94**	**69,870.42**
III. FIXED COSTS	46,693.84	50,938.73	51,193.43
Depreciation and repayment[2]	37,253.47	40,640.15	40,843.35
Taxes	6,293.58	6,865.72	6,900.05
Insurance	3,146.79	3,432.86	3,450.03
IV GENERAL COSTS OF PROCESS	29,544.44	30,574.30	35,190.09
V. OTHER INDIRECT COSTS	-	-	-
INDIRECT COST OF OPERATION	**76,238.28**	**81,513.03**	**86,383.52**
Initial and final inventory of operation	-	-	-
OPERATING COSTS	**134,988.12**	**142,436.97**	**156,253.94**

[1] assuming 55 runs per year

[2] assuming 20% of the total direct cost of the plant, except in buildings, yards preparation and soil (5% were considered), 10% of total indirect costs, and 10% of costs that are not of the plant

Table 7.3 (continuation) Effect of gas blending and feeding strategy on the final production cost of a fermentation process to produce Fab'

Component	Fed-batch (non-GB)	Fed-batch (GB)	pH-stat (GB)
VI. OPERATION EXPENSES[1]	-	-	-
VII. R & D COSTS	24,620.37	25,478.58	29,325.08
GENERAL EXPENSES	**24,620.37**	**25,478.58**	**29,325.08**
VIII. UNEXPECTED[2]	31,921.70	33,583.11	37,115.80
TOTAL PRODUCTION COST	**191,530.19**	**201,498.67**	**222,694.82**
COST OF Fab' (£/L)	12,768.68	13,433.24	14,846.32
Fab' PROCESS YIELD (mg/L)[3]	2200	5500	11000
FINAL PRODUCTION COST OF Fab' (£/mg)	**5.80**	**2.44**	**1.35**

[1] administrative operation expenses were not considered

[2] assuming 3% of the process cost and general costs

[3] assuming 55 runs per year for a batch-fed and pH-stat fermentation (considering 2-3 days of fermentation process and 2-3 days of DSP in both strategies)

Chapter 8 Conclusions and recommendations for Future work

CONCLUSIONS

During this project, a gas blending technique and a pH-stat feeding strategy were proposed to successfully address the problem of O_2 limitation and glycerol oscillations observed in a fermentation process to produce Fab' antibody fragments by *E. coli* W3110 (pAC tAC 4D5 Fab'). Consequently, a method to improve the production of Fab' fragments is claimed to have been developed. Design of experiments methodology (DoE) and regime analysis were utilised for evaluating the effects and interactions of gas blending and feeding strategy on this fermentation process. Studies in a non-gas blending system and in a gas-blending system were carried out. Furthermore, two different feeding strategies were evaluated in a gas blending system, and a preliminary economic analysis was performed. The following sections describe each one of the conclusions in turn.

NON-GAS BLENDING SYSTEM STUDIES

Initially, a batch-fed fermentation process without gas blending was characterised. Results suggested that this fermentation strategy was inefficient at both 20 L and 450 L scale.

Results at 450 L scale indicated total Fab' levels of 75 mg/L, which represents 0.0018 mg of Fab' $L^{-1}g^{-1}$ of lactose consumed. This value was found to be ~95% less when compared to a total Fab' yield of 35 mg/L at 20 L scale, which represents 0.055 mg of Fab' $L^{-1}g^{-1}$ based on lactose utilised.

Based on characteristic times, it was suggested that oxygen limitation most likely occurs at both scales. The time for oxygen consumption (t_{OC}) was less than the time of oxygen transfer (t_{OT}) at all times. By contrast, fermentations with gas blending displayed no significant difference between t_{OC} and t_{OT} and therefore, oxygen limitation is not likely. As a result, a gas blending system at 20 L scale was employed to appropriately maintain constant DOT levels throughout the fermentation process.

GAS BLENDING SYSTEM STUDIES

A factorial 2^2 experimental design was employed to evaluate independently the effects and interactions of two main engineering factors related to oxygen and nutrient transfer in the broth: DOT level and agitation rate. A total Fab' yield of ~140 mg/L was obtained at low agitation rate (500 rpm) regardless of the DOT level. DoE analysis suggested a significant effect of agitation rates and a linear model was proposed as a first approximation to predict Fab' yields in this fermentation process.

The effect of agitation rate was found to be related to the glycerol levels at the time of induction. Fermentations with high agitation rate showed higher levels of glycerol at the point of lactose addition. Based on the K_La values, it was suggested that differences in glycerol levels could be due to better mass transfer conditions that caused the carbon flux to exceed the biosynthetic demands of the cells leading to by-product formation. Final acetate levels of 1-2 g/L and 2.5-5 g/L were measured al low and high agitation rate, respectively.

Furthermore, reduced growth was observed under conditions of high agitation rate and high DOT level. Therefore, it is also suggested that an inhibitory effect on fermentation performance is caused by the high transfer rate of O_2 into the cell in addition to the high transfer rate of other nutrients.

It can be concluded that both glycerol and oxygen transfer will be important parameters to evaluate for the optimisation of this Fab' fermentation. It seems to be a delicate balance to maintain adequate levels of oxygen and the main carbon source flux available for the cell.

FEEDING STRATEGIES STUDIES

In order to obtain higher levels of biomass, two important parameters related to cell growth were investigated. The effects of temperature and concentration of nutrients in the media on biomass levels was evaluated using a 2^2 factorial design. Results showed that a high concentration of nutrients resulted in higher cell densities in shake flasks as expected. Nevertheless, reduced growth and a decrease in product formation were observed in batch-fed fermentations at 20 L scale most probably due to the high acetate levels measured (> 7g/L). This could be explained by the high glycerol uptake rates observed. Fermentations with a two fold concentration of nutrients observed doubling in GUR values when compared with fermentations with normal concentration of nutrients. Alternatives to solve this problem are discussed later.

A problem of glycerol oscillations due to the batch-fed feeding strategy, either with normal or double concentration of nutrients, was identified based on the substrate utilisation profiles. In addition, when comparing fermentations with normal and double nutrient concentrations, results suggested that high levels of glycerol at the time of induction could be limiting product formation.

Therefore, it can be concluded that the presence of glycerol in the induction phase leads to partial suppression of inducer consumption. Examples in the literature suggest that this could be related to the generation of plasmid-free cells and the additional metabolic burden placed on the cells (Lee et al, 1987; Bentley et al, 1989; Glick, 1995).

A pH-stat feeding strategy was proposed to address successfully the problem of glycerol oscillations and reduce glycerol levels at the point of induction. Results showed a total Fab' yield of up to ~200 mg/L and low acetate levels of < 2 g/L. By comparison, a 2-fold increase in the production of Fab' compared to that obtained using batch-fed feeding alone was observed. Product localisation in the cell periplasm of >90% was also achieved.

Based on the pH and OUR profiles, it is suggested that a complete switch of metabolism to lactose cannot be achieved until full depletion of glycerol in the broth has been reached. Results showed that lactose is consumed at a lower rate than glycerol when both are in the broth. The OUR profiles showed that batch-fed fermentations presented diauxic growth in two situations: at the induction point and at the time of glycerol depletion. In addition, a pH switch was only observed after full depletion of glycerol.

Batch-fed fermentations showed higher levels of glycerol at the moment of induction in contrast to that of pH-stat fermentations after induction. This difference could have affected the fermentation performance with regard to acetate formation and lactose utilisation and thus could have caused an additional strain on the cells. Furthermore, the results found in the pH-stat fermentation might be due to the fact that a pseudo-stationary phase with constant cell concentration was reached by the time of the second lactose addition and therefore more C/energy source were available for product formation as opposed to biomass synthesis. The onset of this phase is likely to be the result of phosphate limitation at the time of the second lactose addition.

ECONOMIC CONSIDERATIONS

Economic analysis indicated the potential for the commercial application of a pH-stat fermentation process using a gas blending system. A fermentation production cost of ~£1.45/mg of Fab' was estimated. This resulted in cost savings of up to 75% when compared to the production cost of Fab' in the batch-fed non-gas blending system (~£5.80/mg).

Overall, a pH-stat fermentation process in a gas blending system to produce Fab' fragments is proposed. This fermentation strategy was observed to yield higher Fab' titres compared to traditional batch-fed fermentation in a non-gas blending system and the commercial application seems to be viable. Another advantage of the method developed is its simplicity, as it is coupled the generation of H^+ during the growth phase and pH control with the feeding of the main C-source.

Based on the results obtained, it could be implied that a non-limited O_2 environment under appropriate K_La conditions without glycerol oscillations enhances Fab' titres. Nevertheless, as discussed before, many different factors are related to the performance of this Fab' fermentation. Hence, it is not easy to identify all the effects and interactions between them on product formation alone. Further work is needed, particularly in the induction phase, to investigate the effect and interactions of levels of key nutrients and other operational parameters on Fab' production. Some suggestions are discussed in the following section.

RECOMMENDATIONS FOR FUTURE WORK

Based on the results obtained and on the analysis carried out during this project, the following studies regarding biomass levels, large scale, post-induction, phosphate levels, acetate levels, oxygen supply considerations, and modelling, are discussed for future work.

BIOMASS LEVELS STUDIES

It has previously being discussed in Chapter 6 that doubling concentration of nutrients in shake flasks led to higher levels of biomass. Nevertheless, it is possible that higher acetate levels were inhibiting growth at 20 L scale. Considering this issue and the fact that very low levels of acetate were observed in the pH-stat fermentation it is recommended to run a pH-stat fermentation with double nutrient concentration (except glycerol) to evaluate whether another nutrient might be limiting biomass accumulation.

Based on the results obtained, more experiments could follow towards optimisation of the media components using DoE methodology. For example, the fed-batch culture for the recombinant protein β-Gal production has been reported to be optimised by response surface methods (Chan et al, 1998).

LARGE SCALE STUDIES

In order to evaluate the possibility of a pH-stat process in a gas blending system, an evaluation on a larger scale would be very useful. Therefore, it is recommended to run a pH-stat fermentation using gas blending in order to perform scale-up studies. Unfortunately, the 450 L scale fermenter at UCL is not equipped for gas blending control. A constant mixture of O_2-air in the inlet gas could be used instead. Nevertheless, major issues regarding safety and cost would have to be considered first. Another technical difficulty to overcome at 450 L scale would be the addition of glycerol for the pH-stat feeding strategy. The addition of base is done by over-pressure of the container; therefore, a second pump cannot be coupled as in the 20 L scale. A separate system of pH-control with its pump could be used for the additions of base/acid. Glycerol additions could be done on a second independent pump controlled by the same controller as the base.

The pH-stat fermentation at 450 L scale would be attempted to be run at similar K_La values than at 20 L scale. This could allow a direct comparison with the small scale. The initial target of the experiment would be to fully characterise the fermentation regarding substrate utilisation, product and by-product formation, and characteristic times. It is expected that the findings could lead to further studies on specific key variables to correlate large and small scale towards optimisation of the process.

POST-INDUCTION STUDIES

As previously discussed, it was suggested that biomass formation in the induction phase could be related to the metabolic load of the cells. Therefore, evaluation of different post-induction feeding strategies to control the growth rate after induction in order to increase Fab' yields could be sensible. For example, Lim et al, 1998 have reported that specific growth rate during the post-induction phase could be correlated to production rate in a recombinant *E. coli* fermentation. Results indicated a 23-fold increase in production under an optimal operational strategy. Thus, post-induction feeding to achieve appropriate lactose uptake rates could determine if Fab' yields obtained in pH-stat fermentations could be further increased.

PHOSPHATE LEVELS STUDIES

As already discussed in Chapter 6, a dramatic reduction of phosphate levels has been observed in the induction phase. Therefore, it could be suggested that low levels or depletion of phosphate during the induction phase could increase Fab' productivity. A pump feed rate could be estimated, based on phosphate utilisation profiles, in order to keep minimum constant phosphate levels in the induction phase.

No biomass growth should be observed as phosphate is of central importance for biomass synthesis. Nevertheless, sufficient phosphate needs to be available for the transport of lactose into the cells. A test of this hypothesis could be done by comparison of fermentations with and without constant levels of phosphates during the induction period.

ACETATE LEVELS STUDIES

Although the problem of high acetate levels has partly been solved by a proper feeding strategy, an alternative is discussed to reduce acetate levels and re-evaluate if high levels of biomass could be achieved by doubling the nutrient concentrations.

According to examples in the literature, acetic acid formation could be either the result of lowering the anabolic demand or by enhancing the maximum capacity of the oxidative metabolism. Han et al (1992) have reported successful reduction of specific acetic acid formation by addition of yeast extract and methionine to a 2 L fermentation using glucose as the main C-source. Addition of yeast extract reduced anabolic requirements by supplying cell constituents and thus reduced the glucose uptake rate. On the other hand, a maximum oxidative capacity was observed by addition of methionine attributed to increase the specific oxygen uptake rate of the culture. Therefore, this approach could be used in this Fab' fermentation and re-evaluate increasing the concentration of nutrients to achieve a higher cell density.

OXYGEN SUPPLY CONSIDERATIONS

Simple oxygen enrichment techniques at large scale are usually performed by adding O_2 directly to the air stream prior to the sterile air filter. This allows a higher equilibrium driving force for the O_2 transfer without high capital investment. Nevertheless, the dissolution efficiency of oxygen in enriched air is just as poor as that of an air sparging system.

Direct oxygen injection techniques have been developed to improve oxygen utilisation efficiency. For example, Praxair patented a method to directly inject O_2 into the broth, separated from air. They argued that since pure O_2 bubbles have an oxygen concentration that is five times higher than that of air, very high oxygen dissolution could be achieved (Cheng, 1998). Informing such discussions are results obtained in the gas blending system which suggest a possible detrimental effect of high levels of oxygen and nutrient uptake on biomass levels (see section 5.3.1). Therefore, appropriate levels of O_2 injection need to be evaluated.

MODELLING

A number of biological and non-biological factors have been reported to be related to antibody fragment production (Harrison et al, 1996). Modelling of these factors in order to predict and improve fermentation performance is considered very valuable. Nevertheless, conventional practice of single factor optimisation does not evaluate the interactions present, neither determine the true optimum levels. These disadvantages can be eliminated by collective optimisation using statistical design of experiments. For instance, medium constituents and fermentation conditions (e.g. temperature, pH and the time of fermentation) for the production of L-glutamic acid by *Micrococcus glutamicus* and *Pseudomonas reptilivora* has been reported to be optimised using this kind of approach (Sunitha et al, 1998).

Including characteristic times for modelling that are strongly dependant on fermentation conditions could provide a very useful criterion for scaling-up of fermentation processes. For example, influence of these factors on mixing efficiency for stirred reactors of *Propionibacterium shermanii, Saccharomyces cerevisiae*, and *Penicillium chrysogenum* were evaluated to propose a correlation using a multiregression analysis method (Oniscu et al, 2002). A different approach recently reported for modelling is integrating fluid mixing and microbial kinetics to simulate microbial responses to local oscillations in the environment. Vrábel et al, 2001 developed a predictive model for an *E. coli* fed-batch fermentation using a two compartment scale-down reactor with a mixing time of 112 s. The flow model was verified by mixing time experiments at different regimes from 8 to 22 m^3 scale.

For this project, a group of experiments towards developing a model to evaluate effects and interactions between characteristic times and biological/non-biological factors on Fab' yields could be performed. A factorial design could be used to evaluate effects and interaction of factors such as: oxygen consumption time, oxygen transfer time, agitation rate, inlet oxygen concentration, and feeding rate of lactose on Fab' yields. Initially, 32 experiments would be necessary if evaluation at two levels is carried out. Selection of levels would be based on preliminary experiments to choose an appropriate range of variables. This selection could be done by examining fermentation performance using substrate, by-product and product profiles. Once the factorial design is completed, a response surface technique could be used towards optimisation and validation of the model.

The utilisation of a different approach by means of DoE and regime analysis to evaluate effects and interactions of gas blending and pH-stat feeding strategy was found to be very useful to gain better understanding of this process. Future work has been proposed towards the development and validation of a scale-up model for the optimisation of the process.

References

- Akesson, M., Hagander, P., Axelsson, J.P. Probing control of fed-batch cultivations: analysis and tuning. *Control Eng. Practice.* **2001**, 9: 709-723.
- Alexeeva, S., Hellingwerf, K.J., Teixeira de Mattos, M.J. Quantitative assessment of oxygen availability: perceived aerobiosis and its effect on flux distribution in the respiratory chain of *Escherichia coli*. *J. Bacteriol.* **2002**, 184 (5): 1402-6.
- Amanullah, A., Baba, A., McFarlane, C.M., Emery, A.N., Nienow, A.W., Biological Models of Mixing Performance in Bioreactors. 3rd *International Conference on Bioreactor and Bioprocess Fluid Dynamics*. **1993**, 381-400.
- Amanullah, A., Tuttiet, B., Nienow, A.W. Agitator speed and dissolved oxygen effects in Xanthan fermentations. *Biotech. Bioeng.* **1998**, Vol. 57 2:198-210.
- Andersson, L., Yang, S., Neubauer, P., Enfors, S.-O. Impact of plasmid presence and induction on cellular response in fed-batch cultures of *Escherichia coli*. *J. Biotechnol.* **1996**, 46: 255-263.
- Andersson, L., Strandberg, L., Enfors, S.O. Cell segregation and lysis profound effects on the growth of Escherichia coli in high cell density fed batch cultures. Biotechnol. Prog. **1996b**, 12:190-195.
- Aristidou, A.A.., San, K.Y., Bennett, G.N. Metabolic engineering of *Escherichia coli* to enhance recombinant protein production through acetate reduction. *Biotechnol Prog.* **1995**, 11(4):475-8

- Bailey, J.E., Ollis, D.F. Biochemical engineering fundamentals. 2nd edition, McGraw-Hill International editions, Singapore, **1986**.
- Baneyx, F. Recombinant Protein Expression in *Escherichia coli.* Curr. Opin Biotechnol. 1999, 10:411-421.
- Baykov, A. A., Evtushenko, O. A., Avaeva, S. M. A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. *Anal. Biochem.* **1988,** 171: 266-270.
- Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R., Kompala, D. S. Plasmid-encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria. *Biotech. and Bioeng.*, **1990**, Vol. 35: 668-681.
- Berry, M.J., T.A.K. Wattam, J., Willets, N., Linder, T., De Graaf, T., Hunt, M., Gani, P.J., Davis & P. Porter. Assay and Purification of Fv Fragments in Fermenter Cultures: Design and Evaluation of Generic Binding Reagents. *J. Immunol. Methods.* **1994**, 167: 173-182.
- Better, M. & Horowitz, A.H. Expression of Engineered Antibodies and Antibody Fragments in Microorganisms. *Methods Enzymol.* **1989,** 178: 476-496.
- Better, M., Lin, Y.L. Expression and Scale-up of Engineered Antibody Fragments from *Escherichia coli. Abstracts of Papers of the American Chemical Society.* **1991**, Vol. 202, pp 58-biot.
- Bhattacharya, S. K., Dubey, A. K. Effects of dissolved oxygen and oxygen mass transfer on overexpression of target gene in recombinant *E. coli. Enzyme Microb. Technol.* **1997**, 20: 355-360.

- Bird, R.E., Walker, B.W. Single chain antibody variable regions. *Trends in Biotechnology*. **1991**, 9: 132-137.

- Bowering, L.C. An Engineering Study of the Design, Integration and Control of Antibody Fragment Production Processes. PhD Thesis, University College London, **2000**.

- Bowering, L.C., Bracewell, D.G., Keshavarz-Moore, E., Hoare, M., Neil, A., Weir, C. Comparison of techniques for monitoring antibody fragment production in *E. coli* fermentation cultures, *Biotechnol. Prog.* **2002**, 18:1431-1438.

- Brekke, O.H., Loset, G. A. New technologies in therapeutic antibody development. Current Opinion Pharmacology. **2003**, 3:544-550.

- Bryant, J. The Characterization of Mixing in Fermenters. *Adv. Biochem. Eng.*, 1977, 5:101-123.

- Buchner, J. & Rudolph, R. Renaturation, Purification, and Charachterization of Recombinant Fab Fragments produced in *Escherichia coli. Bio/Technology*. **1991**, 9: 157-164.

- Bylund, F., Collet, E., Enfors, S.-O., Larsson, G. Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation. *Bioprocess Eng.* **1998**, 18: 171-180.

- Bylund, F., Guillard, F., Enfors, S. –O., Trägardh, C., Larsson, G. Scale down of recombinant protein production a comparative study of scaling performance. *Bioprocess Eng.* **1999**, 20: 377-389.

- Bylund, F., Castan, A., Mikkola, R., Veide, A., Larsson, G. Influence of scale-up on the quality of recombinant human growth hormone. *Biotech. & Bioeng.* **2000**, Vol. 69, no. 2, 119-128.

- Cabilly, S. Growth at Sub-optimal Temperatures allows the Production of Functional, Antigen-Binding Fab Fragments in *Escherichia coli*, *Gene.* **1989**, 85:553-557.

- Ca°Caval, D., Oniscu, C., Galaction A., Dumitri, I.F. Characterisation of mixing efficiency in bioreactors. *Roum. Biotechnol. Lett.* **2001**, 6:281-291.

- Carter, P., Kelly, R.F., Rodriguez, M.L., Snedcor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourrel, J.H., Shepard, H.M., Henner, D. High level *E. coli* expression and production of a bivalent huminizes antibody fragment. *Bio/Technology.* **1992**, 10: 163-167.

- Chan, L.C., Greenfield, P.F., Reid, S. Optimising fed-batch production of recombinant proteins using the baculovirus expression vector system. *Biotechnol Bioeng.* **1998**, 59(2):178-88.

- Chen, W., Graham, C., Ciccarelli, R.B. Automated fed-batch fermentation with feed-back controls based on dissolved oxygen (DO) and pH for production of DNA vaccines. *J. of Ind. Microbiol. and Biotech.* **1997**, 18, 43-48

- Cheng, A. How to make oxygen economical for fermentation. *Pharmaceutical ingredients worldwide (CPhI) conference*, Netherlands, **1998**

- Cheng, L., Wu, J., Chen, T. A pseudo-exponential feeding method for control of specific growth rate in fed-batch cultures. *Biochem. Eng. J.* **2002**, 10: 227-232.

- Cui, Y.Q., Van der Lans, R.G.J.M., Noorman, H.J., Luyben, K.C.A.M. Compartment Mixing Model for Stirred Reactors with Multiple Impellers. *Trans IChemE.* **1996**, 74:261-271.

- Dehghani, M., Bulmer, M., Gregory, M.E., Thornhill, N.F. Measurement Variability Analysis for Fermentation Process Assays, *Bioproc. Eng.* **1995**, 13: 239-243.

- De Leon, A., Hernandez, V., Galindo, E., Ramirez, O.T. Effects of dissolved oxygen tension on the production of recombinant penicillin acylase in *Escherichia coli. Enzyme and Microb. Tech.* **2003**, 33: 689-697.

- Di Luccio, M., Borges, C.P., Alves, T.L.M. Economic analysis of ethanol and fructose production by selective fermentation coupled to pervaproation: effect of membrane costs on process economics. *Desalination.* **2002**, (147) 161-166.

- Donovan, R.S., Robinson, C.W., Glick, B.R. Optimizing the expression of a monoclonal antibody fragment under the transcriptional control of the *E. coli* lac promoter. *Can J Microbiol.* **2000**, 46(6): 532-541.

- Doran, P.M. Bioprocess Engineering Fundamentals. Academic Press Inc., Great Britain, **1995.**

- Dunn, I.J., Mor, J.-R. Variable-volume continuous cultivation. *Biotecnol Bioeng.* **1975**, 17:1805-1822.

- El-Mansi, E.M., Holms, W.H. Control of carbon flux to acetate excretion during growth of Escherichia coli in batch and continuous cultures. *J Gen Microbiol.* **1989**, 135 (11):2875-83.

- Farewell, A., Neidhardt, F. Effect of temperature on *in vivo* protein synthetic capacity in *Escherichia coli. J. of Bacteriology*, **1998**:4704-4710.

- Ferguson, G.P. Tötemeyer, S., MacLean, M.J., Booth, I.R. Methylglyoxal production in bacteria: suicide or survival? *Arch. Microbiol.* **1998**, 170: 209-219.

- Flores, F., Torres, L.G., Galindo, E. Effect of the dissolved-oxygen tension during cultivation of *Xanthomonas campestris* on the production and quality of Xanthan gum. *J. Biotech.* **1994**, Vol. 34 2:165-173.

- Flores, S., Anda-Herrera, R., Gosset, G. Growth-rate recovery of Escherichia coli cultures carrying a multycopy plasmad, by engineering of the pentose-phosphate pathway, *Biotechnol and Bioeng.*, **2004,** Vol. 87: 485-494.

- Frenken, L.G.J., Hessing, J.G.M., Van den Hondel, C.A.M.J.J., Verrips, C.T. Recent Advances in the Large-scale Production of Antibody Fragments using Lower Eukaryotic Microorganisms, *Research in Inmunology.* **1998**, Vol. 149, 6:589-599.

- Garcia Arrazola, R., R. Rodriguez Vazquez, E.J. Manrique Ramirez, R.J. Garcia Barajas, D. Diaz Cervantes, and J.M. Fernandez Sanchez. Bioprocess of Wastewater Aromatic Dyes: Cost and Significant Variables. *Proceedings of the Sixth International In Situ and On-Site Bioremediation Symposium, Ex Situ Biological Treatment Technologies.* **2001,** Vol. 6(6). pp. 369-376 Battelle Press, Columbus, Ohio.

- Garret, D.E. Chemical Engineering Economics, V.N. Reinhold, New York, **1989.**

- Gibbs, P.A., Seviour, R.J. Does agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aurebasidium pullulans* in batch culture? *Appl.Microbiol Biotechnol.* **1996**, 46: 503-510.

- Giridhar, R., Srivastava, A. Computer coupled substrate feeding strategies for efficient conversion of D-sorbitol to L-sorbose in fed-batch culture. *Process Biochemistry.* **2001**, 36: 829-834.

- Glick, B. Metabolic load and heterologous gene expression. *Biotech Advances*, **1995**, Vol. 13: 247-261

- Glockshuber, R., Malia, M., Pfitzinger, I., Pluckthun, A. A comparison of strategies to stabilise immunoglubilin Fv fragments. *Biochmistry.* **1990**, 29:1362-1367

- Gogate, P. R., Beenackers, A. A. C. M., Pandit, A.B. Multiple-impeller systems with a special emphasis on bioreactors: a critical review. *Biochem. Eng. J.* **2000**, 6: 109-144.

- Guardia, M.J., García-Calvo, E. Modelling of *Escherichia coli* growth and acetate formation under different operational conditions. *Enzyme and Microbial Technology*, **2001**. 29: 449-455

- Guillard, F., Trägardh, C. Mixing in industrial Rushton turbine-agitated reactors under aerated conditions. *Chem. Eng. and Proc.*, **2003.** 42:373-386

- Gupta, A., Rao, G. A study of oxygen transfer in shake flasks using a non-invasive oxygen sensor. *Biotechnol Bioeng.* **2003**, 5;84(3):351-8.

- Hamidi-Esfahania, Z., Shojaosadatia,S.A., Rinzema. A. Modelling of simultaneous effect of moisture and temperature on *A. niger* growth in solid-state fermentation. *Biochem Eng. J.* **2004**, 21:265–272.

- Han, K., Lim, H., Hong, J. Acetic acid formation in Escherichia coli fermentation. *Biotechnol. Bioeng.*, **1992**, Vol 39: 663-671.

- Harris, B. Exploiting Antibody-based Technologies to Manage Environmental Pollution. *Trends in Biotech.* **1999**, Vol. 17, 7:290-296.

- Harrison, J.S., Keshavarz-Moore, E. Production of Antibody Fragments in Escherichia coli. *Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences.* **1996**, Vol. 182.

- Hewitt, C., Nebe-Von Caron, G., Axelsson, B., McFarlane, C.M., Nienow, A.W. Studies related to the scale-up of high-cell density E. coli fed-batch fermentations using multiparameter flow cytometry: Effect of changing microenvironment with respect to glucose and dissolved oxygen concentration. *Biotech. and Bioeng.* **2000**, Vol 70, num 4, 381-389.

- Hogema, B.M., Arents, J.C., Baden, R., Postma, P. Autoregulation of lactose uptake through the LacYpermease by enzyme IIAGlc of the PTS in Escherichia coli K12. *Molec. Microb.* **1999**, 31: 1825-1833.

- Holms, W.H. The central metabolic pathways of *E. coli* relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion to acetate. *Curr. Top. Cell.* **1986** Reg. 28 69-105.

- Holms, W.H. Flux analysis and control of the central metabolic pathways in *E. coli*. *Microb. Rev.* **1996**, 19: 85-116.

- Horn, U., Strittmatter, W., Krebber, A., Knupfer, U., Kujau, M., Wenderoth, R., Muller, K., Matzku, S., Pluckthun, A., Riesenberg, D. High volumetric yields of functional dimeric antibodies miniantibodies in *Escherichia coli*, using and optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl. Microbiol. Biotechnol.* **1996**, 46, 524-532.

- Hsu, Y., Wu, W. A novel approach for scaling-up a fermentation system. *Biochem. Eng. J.* **2002**, 123-130.

- Hudson, P.J. Recombinant antibody constructs in cancer therapy. *Current Opinion in Immunology.* **1999**, 11: 548-557.

- Humphreys D.P., Carrington, B., Bowering, L.C., Ganesh, R., Sehdev, M., Smith, B.J., King, L.M., Reeks, D.G., Lawson, A., Popplewell, A.G. A plasmid system for optimization of Fab' production in *Escherichia coli*: importance of balance of heavy chain and light chain synthesis. *Protein Expr Purif.* **2002**, 26(2):309-20.

- Inbar, D., Hochman, J., Givol, D. Localisation of antibody-combining sites within the variable protions of heavy and light chains. *Proceedings of the National Academy of Sciences, USA.* **1972**, 69:2659-2662.

- Jensen, E.B., Carlsen, S. Production of recombinant human growth hormone in *Escherichia coli* : Expression of different precursors and physiological effects of glucose, acetate and salts. *Biotechnol. Bioeng.* **1990**. 36:1-11

- Johnston, W., Cord-Ruwisch, R., Cooney, M.J. Industrial control of recombinant *E. coli* fed-batch culture: new perspectives on traditional controlled variables. *Bioprocess Biosystems Eng.* **2002**, 25: 111-120.

- Kasemets, K., Drews, M., Nisamednitov, I., Adamberg, K., Paalme, T. Modification of A-stat for the characterisation of microorganisms. *J. Microb. Methods.* **2003**, 55:187-200.

- King, D.J., Byron, O.D., Mountain, A., Weir, N., Harvey, A., Lawson, D.G., Proudfoot, K.A., Baldock, D., Harding, E., Yarranton, G.T., Owens, R.J. Expression, purification and characterisation of B72.3 Fv fragments. *Biochem J.* **1993**, 290: 723-729

- Kipriyanov, S.M., Moldenhauer, G., Little, M. High Level Production of Soluble Single Chain Antibodies in Small-scale Escherichia coli cultures, *Immunological Methods*. **1993**, Vol. 200, 1-2:69-77.

- Kipriyanov, S.M., Moldenhauer, G., Braunagel, M., Reusch, U., Cochlovius, B., Le Gall, F., Kouprianova, O.A., Von der Lieth, C., Little, L. Effect of Domain Order on the Activity of Bacterially Produced Bispecific Single-chain Fv Antibodies, *J. Mol. Biol.* **2003**, 330, 99–111.

- Kleist, S., Miksch, G., Hitzmann, B., Arndt, M., Friehs, K., Flaschel, E. Optimization of the extracellular production of a bacterial phytase with *Escherichia coli* by using different fed-batch fermentation strategies. Appl. Microbiol Biotechnol. **2003**, 61:456-462.

- Korz, D.J., Rinas, U., Hellmuth, K., Sanders, E. A., Deckwer, W.-D. Simple fed-batch technique for huigh cell density cultivation of *Escherichia coli. J. Biotech.* **1995**, 39: 59-65.

- Larsson, G., Törnkvist, M., Stahl Wernesson, E., Trägardh, C., Noorman, H., Enfors, S-O. Substrate Gradients in Bioreactors: Origin and Consequences. *Bioprocess Eng.* **1996**, 14:281-289.

- Larsson, G. & Enfors, S-O. Studies of Insufficient Mixing in Bioreactors: Effect of limiting Oxygen Concentrations and short term Oxygen Starvation on *Penicillum chrysogenum. Bioprocess Eng.*, **1998**, 3:123-127.

- Lee, A.T., Cerami, A. Elevated glucose 6-phosphate levels are associated with plasmid mutations *in vivo. Proc. Natl. Acad. Sci. USA.* **1987**, 84:8311-8314

- Lee S.Y. High Cell Density Culture of *Escherichia coli. Trends Biotechnol.* **1996**, 14: 98-105.
- Leser, E.W., & Asenjo, J.A. Large-scale Protein Purification. In Harris, E.L.V. & Angal, S., Protein Purification Applications: a Practical Approach (pp 1-28). Oxford: IRL Press, **1992.**
- Li X., Robbins, J.W. Jr., Taylor, K.B. Effect of the levels of dissolved oxygen on the expression of recombinant proteins in four recombinant *Escherichia coli* strains. *J. Ind. Microbiol.* **1992**, 9 (1): 1-9.
- Li, Y., Chen, J., Mao, Y., Lun, S., Koo, Y. Effect of additives and fed-batch culture strategies on the production of glutathione by recombinant *Escherichia coli. Process Biochemistry.* **1998**, Vol. 33, No. 7, 709-714.
- Lienqueo, M.E., Asenjo, J.A. Use of expert for the synthesis of downstream protein processes. *Computers and Chemical Engineering.* **2000**, 24: 2339-2350.
- Lim, A.C., Zhou, Y., Washbrook, J., Titchener-Hooker, N.J., Farid, S. A decisional-support tool to model the impact of regulatory compliance activities in the biomanufacturing industry. *Comp. and Chem. Eng.*, **2004**, 28: 727-735.
- Lim H.K., Jung, K.H. Improvement of heterologous protein productivity by controlling postinduction specific growth rate in recombinant *Escherichia coli* under control of the PL promoter. *Biotechnol Prog.* **1998,** 14(4):548-53.

- Lin, H.Y., Neubauer, P., Influence of controlled glucose oscillations on a fed-batch process of recombinant Escherichia coli. *J. of Biotech.* **2000**, 79: 27-37.

- Lodish, H., Berk, A., Lawrence Zipursky, S., Matsudaira, P., Baltimore, D., Darnell, J. Molecular cell biology, 4th edition, Freeman and Company, New York, **2000**.

- Luli, G., Strohl, W.R., Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *App. and Env. Microbiol.* **1990**, Vol. 56, No.4, p. 1004-1011.

- Maass, D., Gerigk, M.R., Kreutzer, A., Weuster-Botz, D., Wubbolts, M., Takors, R. Integrated L-phenylalanine separation in an *E. coli* fed-batch process: from laboratory to pilot scale. *Bioprocess Biosyst. Eng.* **2002**, 25: 85-96.

- Macaloney, G., Hall, J. W., Rollins, M.J., Draper, I., Anderson, K.B., Preston, J., Thompson, B.G., McNeil, B. The utility and performance of near-infra red spectroscopy in simultaneous monitoring of multiple components in a high cell density recombinant *Escherichia coli* production process. *Bioproc. Eng.* **1997**, 17:157–167

- Manna, L. Comparison between physical and chemical methods for the measurement of mixing times. *Chemical Eng. J.* **1997**, 67: 167-173.

- Michel, B.J., Miller, S.A. Power requirements of gas-liquid agitated systems. *A.I.Ch.E. J.*, **1962**, 262-266

- Missiakas, D., Raina, S. Protein misfolding in the cell envelope of *Escherichia coli*: new signalling pathways. *Trends in Biochemical Sciences*. **1997**, 22:59-63

- Moes, J., Griot, M., Keller, J., Heinzle E., Dunn, I.J., Bourne, JR. A Microbial Culture with Oxygen-sensitive Product Distribution as a Potential Tool for characterizing Bioreactor Oxygen Transport., *Biotechnol. Bioeng*. **1985**, 27: 482-489.

- Montgomery, D.C. Design and Analysis of Experiments. 5th edition, John Wiley & Sons, Inc., USA, **2001**.

- Mujacic, M., Cooper, K.W., Baneyx, F. Cold-inducible cloning vectors for low-temperature protein expression in *Escherichia coli*: application to the production of a toxic and proteolytically sensitive fusion protein *Gene*, **1999**; Vol. 238: 325-332

- Mustafa, M.A., Washbrook, J., Lim, A.C., Zhou, Y., Titchener-Hooker, N.J., Morton, P., Berezenko, S., Farid, S. A software tool to assist business-process decision-making in the biopharmaceutical industry. *Biotechnol. Prog*. **2004**, 20:1096-1102.

- Nakano, H. Improvement and applications of cell-free protein synthesis systems. *J. of Bioscience and Bioeng.*, **2003**, Vol. 95: 206-207

- Nawani, N.N., Kapadnis, B.P. Optimization of chitinase production using statistics based experimental designs. *Proc. Biochem*. **2004,** article in press.

- Neubauer, P., Lin, H.Y., Mathiszik, B. Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli. Biotech. and Bioeng.* **2003**, Vol. 83, No. 1, 53-64.

- Nielsen, J., Villadsen, J., Liden, G. Bioreaction Engineering Principles. 2nd edition. Kluwer Academic/Plenum Publishers, **2003**.

- Nikiforaki, L., Yu, J., Baldi, S., Genenger, B., Lee, K.C., Durst, F., and Yianneskis, M. On the variation of precessional flow instabilities with operational parameters in stirred vessels. *Chemical Engineering Journal*, **2004**, Vol. 102, pp. 217-231.

- Novais, J.L., Titchener-Hooker, N.J., Hoare, M. Economic comparison between conventional and disposables-based technology for the production of biopharmaceuticals. *Biotechnology and Bioengineering*. **2001**, Vol. 75(2), 143-153.

- Nygren, P.A., Kondo, A., Persson, B., Uhlen, M. Stability of Antibody Fragments Secreted to the culture medium of *Escherichia coli.*, *Protein Eng.* **1993**, 6(SS):92

- O'Bierne, D., Hammer, G. Oxygen availability and the growth of Escherichia coli W3110: A problem exacerbated by scale-up. *Bioprocess Eng.* **2000**, 23:487-494.

- O'Kennedy, R.D., Baldwin, C., Keshavarz-Moore, E. Effects of growth medium selection on plasmid DNA production and initial processing steps. *J. of Biotech.* **2000**, 76:175-183.

- Oniscu, C., Galaction, A.-I, Cascaval, D., Ungureanu, F. Modelling of mixing in stirred bioreactors 2. Mixing time for non-aerated broths. *Biochem. Eng. J.* **2002**, 12: 61-69.

- Onyeaka, H., Nienow, A. W., Hewit, C. J. Further studies related to the scale-up of high cell density Escherichia coli fed-batch fermentations: the additional effect of a changing microenvironment when using aqueous ammonia to control pH. *Biotechnol Bioeng.* **2003**, 84(4):474-484.

- Oosterhuis, N.M.G., Groebeek, N.M., Olivier A.P.C., Kossen N.W.F. Scale-down Aspects of the Gluconic Acid Fermentation. *Biotechnol. Lett.* **1983,** 5: 141-146.

- Oosterhuis, N.M.G., Kossen, N.W.F., Olivier, A.P.C., Schenk E.S. Scale-down and Optimization Studies of the Gluconic Acid Fermentation by *Gluconobacter oxydans*. *Biotechnol. Bioeng.* **1985**, 27:711-720.

- Pack, P., Kujau, M., Schroeckh, V., Knupfer, U., Wenderoth, R., Reisenberg, D., Pluckthun, A. Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli*. *Bio/Technology*. **1993**, 11: 1271-1277.

- Panuwatsuk, W., Da Dilva, N.A. Application of a gratuitous induction system in for the expression of intracellular and secreted proteins during fed-batch culture. *Biotechnol Bioeng.* **2003**, 81(6):712-718.

- Peters, M.S., Timmerhaus, K.D. Plant design and economics for chemical engineers, USA, **2002.**

- Pierce, J.J., Robinson, S.C., Ward, J.M., Keshavarz-Moore, E., Dunnill, P. A comparison of the process issues in expressing the same recombinant enzyme periplasmically in *Escherichia coli* and extracellularly in *Streptomyces lividans*. *J. of Biotech.* **2002**, 92:205-215.

- Pluckthun, A. Antibody Engineering: Advances from the use of *Escherichia coli* Expression Systems. *Bio/Technology.* **1991**, 9: 545-551.

- Pluckthun, A. Mono- and Bivalent Antibody Fragments Produced in *Escherichia coli*: Engineering, Folding and Antigen Binding. *Inmunol.Rev.* **1992**, 130:151-188.

- Pollard, D. J., Kirschner, T. F.; Hernandez, D., Hunt, G., Olewinski, R., Salmon, P.M. Pilot-scale process sensitivity studies for the scaleup of a fungal fermentation for the production of pneumocandis. *Biotech. & Bioeng.* **2002**, Vol. 78, no. 3, 270-279.

- Porter, R.R. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem J.***1959**. 73:119-126

- Raag, R., Whitlow, M. Single chain Fvs. *FASEB J.* **1995**, 9:73-80

- Reiter, Y., Brinkman, U., Lee, B., Pastan, I. Engineering antibody Fv fragments for cancer therapy: disulfide-stabilised Fv fragments. *Nature Biotechnology.* **1996**, 14: 1239-1245.

- Robbins, J. W., Taylor, K.B. Optimization of *Escherichia coli* growth by controlled addition of glucose. *Biotech. and Bioeng.* **1989**, Vol. 34, 1289-1294.

- Rocha, I., Ferreira, E. C. On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant *E. coli*. *Analytica Chimica Acta*, **2002** 462: 293-304.

- Rocha-Valadez, J.A., Galindo, E., Serrano-Carreón, L. Effect of the impeller-sparger configuration over *Trichoderma harzanum* growth in tour-phases cultures under constant dissolved oxygen. *Bioprocess Engineering*. **2000**, 23: 403-410.

- Roque, A. C., Lowe, C. R., Taipa, M. A. Antibodies and genetically engineered related molecules production and purification. Biotechnol. Prog. **2004**, 20: 639-654.

- Rozkov, A., Enfors, S.-O. Stabilization of a proteolytically sensitive cytoplasmic recombinant protein during transition to downstream processing. *Biotech. & Bioeng.*, **1999**. Vol. 62, No. 6, 731-738.

- Sabatié, J., Speck, D., Reymund, J., Hebert, C., Caussin, L., Weltin, D., Gloeckler, R., O'Regan, M., Bernard, S., Ledoux, C., Ohsawa, I., Kamogawa, K. Biotin formation by recombinant strains of *Escherichia coli*: influence of the host physiology. *Journal of Biotechnology*, **1991**, 20:29-49.

- Sanden AM, Prytz I, Tubulekas I, Forberg C, Le H, Hektor A, Neubauer P, Pragai Z, Harwood C, Ward A, Picon A, De Mattos JT, Postma P, Farewell A, Nystrom T, Reeh S, Pedersen S, Larsson G. Limiting factors in *Escherichia coli* fed-batch production of recombinant proteins. *Biotechnol Bioeng*. **2003**, 20; 81(2):158-66.

- Schilling, B. M., Pfefferle, W., Bachmann, B., Leuchtenberger, W., Deckwer, W.-D. A special reactor design for investigations of mixing time effects in a scale-down industrial L- lysine fed-batch fermentation process. *Biotech. & Bioeng.* **1999,** 64: 599-606.

- Schmiedl, A., Breitling, F., Dubel, S. Expression of a Bispecific dsFv-dsFv' Antibody Fragment in *Escherichia coli. Protein Eng.* **2000**, Vol. 13, 10:725-734.

- Schwarz, L.H., Rudolph, E. Advances in Refolding of Proteins produced in *E. coli. Curr. Opin. Biotechnol.* **1998**, 9: 497-501.

- Shibui,T., Munataka, K., Matsumoto, R., Ohta, K., Matsushima, R., Morimoto, Y., Nagahari, K. High level production and secretion of a mouse chimeric Fab fragment with specificity to human carcino embryonic antigen in *E. coli. Appl. Microbiol. Biotechnol.* **1993**, 38: 770-775.

- Shokri, A., Sandén, A.M., Larsson, G., Growth rate-dependent changes in *Escherichia coli* membrane structure and protein leakage. *Appl. Microbiol. Biotehcnol.* **2002**, 58: 386-392.

- Shukla, V.B., Parasu, U., Kulkarni, P.R., Pandit, A.B. Scale-up of biotransformation process in stirred tank reactor using dual impeller bioreactor. *Biochem. Eng. J.* **2001**, 8:19-29.

- Sivers, V.M., Zacchi, G., Olsson, L., Hahn-Hägerdal, B. Cost analysis of ethanolproduction from willow using recombinant *Escherichia coli. Biotechnol. Prog.* **1994**, 10: 555-560.

- Skerra, A., Pluckthun, A. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli. Science*, **1988**, 240:1038-1041

- Somerville, J.E., Goshorn, S.C., Fell, H.P., Darveau, R.P. Bacterial aspects associated with the expression of a single-chain antibody fragment in *Escherichia coli. App. Microbiol. & Biotech.* **1994**, 42:595-603.

- Sotiriadis, A., Keshavarz, T., Keshavarz-Moore, E. Factors affecting the Production of a Single-chain Antibody Fragment by *Aspergillus awamori* in a Stirred Tank Reactor. *Biotechnol. Prog.* **2001**, Vol. 17, 4:618-623.

- Stanbury, P.F., Whitaker, A., Hall, S.J. Principles of fermentation technology. 2nd edition, Butterworth-Heinemann Ed., UK, **2000.**

- Straight, J., Parulekar, S., Jansen, N. Bacterial growth on lactose: an experimental investigation. *Biotechnol. Bioeng.*, **1989**, Vol: 34: 705-716.

- Strandberg, L., Kohler, K., Enfors, S.O. Large-scale fermentation and Purification of a Recombinant Protein from *Escherichia coli. Proc. Biochem.* **1991,** 26:225-234.

- Strandberg, L., Andersson, L., Enfors, S.O. The use of Fed-batch Cultivation for achieving High Cell Densities in the Production of a Recombinant Protein in *Escherichia coli*", *Proc. Biochem.* **1994**, 26:225-234.

- Sunitha, I., Subba Rao M.V., Ayyanna, C. Optimization of medium constituents and fermentaion conditions fo rthe production of L-glutamic acid by the coimmobilized whole cells of *Micrococcus glutamicus* and *Pseudomonas reptilivora. Bioprocess Eng.* **1998**, 18: 353-359.

- Swartz, J.R. Advances in *Escherichia Coli* Production of Therapeutic Proteins, *Current Opinion in Biotechnology.* **2001**, 12:195-201.
- Sweere, A. P. J., Luyben, K. Ch. A. M., Kossen, N. W. F. Regime analysis and scale-down: tools to investigate the performance of bioreactors. *Enzyme Microb. Technol.* **1987**, vol.9, 386-398.
- Tomlinson, I., Holliger, P. Methods for generating Multivalent and Bispecific Antibody Fragments, *Appl. Of Chimeric Genes and Hybrid Proteins.* **2000**, Vol. 326, 461-479.
- Vaara, M., Agents that increase the permeability of the outer membrane. *Microbiological Reviews* **1992**, 56: 395-411.
- Van't Riet, K. Mass transfer in fermentation. *Trends in Biotech.*, 1983, 1:113-119.
- Voet, D., Voet, J. G. Biochemistry. 2nd edition, USA, **1995**. Weir, A.N.C., Mountain, A. Method for expressing recombinant genes in bacteria in absence of antibiotic selection. *United States Patent*, **1995**. Appl. No. 411778.
- Vrábel, P, van der Lans, R.G.J.M., van der Schot, F.N., Luyben, K.Ch.A.M., Xu, B., Enfors, S.-O. CMA: integration of fluid dynamics and microbial kinetics in modeling of large-scale fermentations. *Chem. Eng. J.* **2001**, 84: 463-474.
- Wang Y., Yang, B., Renb, J., Donga, M., Liang, D., Xua, A. Optimization of medium composition for the production of clavulanic acid by *Streptomyces clavuligerus*. *Proc Biochem.* **2004**, article in press

- Weir, A.N.C., Bailey, N.A. Process for obtaining antibodies utilising heat treatment. United States Patent, 1995. Appl. No. 367313.
- Weir, A.N.C., Mountain, A. Method for expressing recombinant genes in bacteria in absence of antibiotic selection. United States Patent, 1999. Appl. No. 411778.
- Weltey, J.R., Wiggs, C.E., Wilson, R.E., Rorrer, G.L. Fundamentals of momentum, heat and mass transfer. 4th edition, USA, **2001.**
- Wheelwright, S.M. Designing Downstream Processes for Large Scale Protein Purification, *Bio/Technology*. **1987**, 5:789-793.
- Yee, L. & Blanch. Recombinant Protein Expression in High Cell Density Fed-Batch Cultures of *Escherichia coli*. *Bio/Technology*. **1992**, 10: 1550-1556.

Appendix A

The raw data from the fermentation run at 450 L without gas blending (see table A.1) has been used for this sample calculation.

Table A.1 Raw data at 24 h for the fermentation at 450 L scale without gas blending.

time, h	Volume, L	Airflow, Lpm	DOT, %	P_{total}, atm	N_2 (inlet), %	N_2 (outlet), %	O_2 (inlet), %	O_2 (outlet), %
24	300	450.7	0.37	1.1	78.0467	78.8580	20.9686	18.3311

The values for K_La, $t_{(OC)}$, $t_{(OT)}$ were estimated (see table A.2) from the experimental on-line readings summarised in the previous table.

Table A.2. Values of K_La, $t_{(OC)}$, $t_{(OT)}$ at 24 h for the fermentation at 450 L scale without gas blending.

time, h	K_La, h^{-1}	$t_{(OC)}$	$t_{(OT)}$
24	362.62	0.038	9.94

Firstly, the amount of inlet gas to the fermenter was estimated as follows:

$$Gas_{in} = \frac{1}{22.4(\frac{L}{mol})1} * 450.7 \frac{L}{\min} * \frac{1}{300L} 60 \frac{\min}{h} * 1000 \frac{mmoL}{mol} = 4024.07 \frac{mmoL}{L.h}$$

1. Assuming that 1 mol of gas occupies 22.4 L at standard temperature and pressure.

Then, Gas $_{out}$ was estimated as follows:

$$Gas_{out}^{2} = \frac{N_2(outlet)}{N_2(inlet)} * Gas_{in} = \frac{78.8580}{78.0467} * 4024.07 \frac{mmoL}{L.h} = 4065.9 \frac{mmoL}{L.h}$$

2. Assuming that no N_2 was absorbed from the gaseous to the liquid phase.

Afterwards, OUR was estimated as follows:

$$O_2(inlet) = Gas_{in} * \%O_2(inlet) = 4024.07 \frac{mmoL}{L.h} * \frac{20.9686}{100} = 843.79 \frac{mmoL}{L.h}$$

$$O_2(outlet) = Gas_{out} * \%O_2(outlet) = 4065.9 \frac{mmoL}{L.h} * \frac{18.3311}{100} = 745.32 \frac{mmoL}{L.h}$$

$$OUR = O_2(inlet) - O_2(outlet) = 843.79 - 745.32 = 98.47 \frac{mmoL}{L.h}$$

Once the OUR was estimated, the driving force C^*-C_L was estimated as follows:

$$C^*_{O_2(inlet)} = \frac{P_{O_2(inlet)}}{H_{O_2}} = \frac{(C_{O_2inlet})P_{tot}}{H_{O_2}} = \frac{(\frac{20.9686}{100}) * 1.1atm}{790.6 \frac{atm.L}{mol}} = 2.92E-4 \frac{mol}{L}$$

$$C^*_{O_2(outlet)} = \frac{P_{O_2(outlet)}}{H_{O_2}} = \frac{(C_{O_2outlet})P_{tot}}{H_{O_2}} = \frac{(\frac{18.3311}{100}) * 1.1atm}{790.6 \frac{atm.L}{mol}} = 2.55E-4 \frac{mol}{L}$$

$$(C^* - C_L)_{\log mean} = \frac{(C^*_{inlet} - C^*_{outlet})(1 - \%DOT)}{LN\left[\frac{C^*_{inlet}}{C^*_{outlet}}\right]} = \frac{(2.92E-4) - (2.55E-4) * (1 - 0.37)}{LN\left[\frac{2.92E-4}{2.55E-4}\right]} = 2.72E-4 \frac{mol}{L}$$

Finally,

$$K_L a = \frac{qO_2}{(C^* - C_L)} = \frac{98.47(mmoL_{L.h}) * \left(mol_{1000mmoL}\right)}{2.72E-4\, mol_L} = 362.02h^{-1}$$

$$t_{(X)}(s) = \frac{C_{O_2 average}}{r_{O_2}} = \frac{(C_{O_2 inlet} + C_{O_2 outlet})}{rO_2} 2 * DOT = \frac{[(2.92E-4)+(2.72E-4)]}{98.47\, mmoL_{L.h}} 2 * (0.37_{100}) moL_L * \frac{3600s}{1h} = 0.038s$$

$$t_{OT}(s) = \frac{1}{K_L a} = \frac{1}{362.02h^{-1}} * \frac{3600s}{1h} = 9.94s$$

Appendix B

BATCH PRODUCTION RECORD (BPR) TO PRODUCE Fab ANTIBODY FRAGMENTS BY *E. coli* FERMENTATION AT 20 L SCALE

INTRODUCTION

The record is divided in three principal stages:

I. Preinoculation. This stage includes the preparation of the elements required for the fermentation:

a) Materials (reactants and glassware)
b) Solutions (chloramphenicol, $MgSO_4.7H_2O$ and, trace elements)
c) Petri plates (for purity and sterilisation checks)
d) Fermentation ancillaries
e) Complex Media, CM
f) Defined Media (flask), DM(fl)
g) Defined Media (fermenter), DM(fe)
h) Set up of HPLC for reading glycerol consumption

II. Inoculation. This stage includes:

a) The inoculation in CM with a vial from the Working Cell Bank, WCB
b) Inoculation in DM(fl) from the culture in CM
c) Final inoculation into fermenter from the culture in DM(fl)

III. Fermentation. This final stage includes the whole process after inoculation

a) Fermentation process
b) Harvest
c) Analysis of samples and results

I. PREINOCULATION

A) Materials **Date:** _____

Material	Supplier/ Size (g)/ Batch Number
COMPLEX MEDIA:	
1. Tryptone	
2. Yeast Extract	
3. NaCl	
DEFINED MEDIA (flask & fermenter)	
1. $(NH_4)_2SO_4$	
2. NaH_2PO_4	
3. KCl	
4. Citric Acid	
5. Glycerol	
6. $MgSO_4.7H_2O$ (solution)	
TRACE ELEMENTS SOLUTION FOR DEFINED MEDIA (flask and fermenter)	
1. Citric Acid	
2. $CaCl_2.6H_2O$	
3. $ZnSO_4.7H_2O$	
4. $MnSO_4.4H_2O$	
5. $CuSO_4.5H_2O$	
6. $CoSO_4.7H_2O$	
7. $FeCl_3.6H_2O$	
8. H_3BO_3	
9. $NaMoO_4.2H_2O$	
CHLORAMPHENICOL SOLUTION FOR COMPLEX AND DEFINED MEDIA (flask and fermenter)	
1. Chloramphenicol	
2. Ethanol	
LACTOSE SOLUTION FOR FERMENTATION	
1. Lactose	
GRAM STAIN (Petri plates)	
1. Gram Stain	
GLASSWARE	
1. Flasks	
2. Petri plates	
BASIC AND ACID CONTROLERS	
Ammonia Solution (30%)	
Sulphuric Acid	

Initials_____
Initials_____

I. PREINOCULATION

B) Solutions **Date:** _____

1. Make up of Chloramphenicol solution for the flasks and the fermenter

Chloramphenicol solution for Flasks	
A. Chloramphenicol concentration in flask (g/L)	0.025
B. Working Volume of Media in flask (L):	0.25
C. Mass required (g) A x B	0.00625
D. Volume (mL) to add into the media*	0.5
E. $\delta_{solution}$ (g/mL) C / D	0.0125
F. Volume to prepare (mL)*	40
G. To weigh (g) E x F	0.5

Chloramphenicol solution for Fermenter	
A. Chloramphenicol concentration in fermenter(g/L)	0.025
B. Working Volume of Media (L):	12.5
C. Mass required (g) A x B	0.3125
D. Volume (Ml) to add into the media*	25
E. $\delta_{solution}$ (g/Ml) C / D	0.0125
F. Volume to prepare (mL)*	40
G. To weigh (g) E x F	0.5

* controllable variable

Weigh 0.5g of chloramphenicol and dissolve in 40mL of ethanol

Weight (g): _____ V (mL): _____

Initials _____
Initials _____

I. PREINOCULATION

2. Make up of $MgSO_4.7H_2O$ solution for the flasks and the fermenter

$MgSO_4.7H_2O$ for flasks	
A. $MgSO_4.7H_2O$ concentration in flask(g/L)	1
B. Working Volume of Media (L)*:	0.25
C. Mass required (g) A x B	0.25
D. Volume (mL) to add into the media*	2.5
E. $\delta_{solution}$ (g/mL) C / D	0.1
F. Volume to prepare (mL)*	150
G. To weigh (g) E x F	15

$MgSO_4.7H_2O$ solution for fermenter	
A. $MgSO_4.7H_2O$ concentration in fermenter (g/L)	1
B. Working Volume of Media (L)*:	12.5
C. Mass required (g) A x B	12.5
D. Volume (mL) to add into the media*	125
E. $\delta_{solution}$ (g/mL) C / D	0.1
F. Volume to prepare (mL)*	150
G. To weigh (g) E x F	15

* controllable variable

Weigh 15g of $MgSO_4.7H_2O$ and dissolved in 150mL R.O. water.

Weight (g): _____ V (mL): _____

Initials_____
Initials_____

Autoclave the solution according to SOP of ACBE

I. PREINOCULATION

3. Make up of one Trace Elements solution for the flasks and the fermenter.

Compound	Concentration, C	To weigh (g) C x V	Weigh (g)
Citric Acid (g/L)	100		
$CaCl_2.6H_2O$ (g/L)	5		
$ZnSO_4.7H_2O$ (g/L)	2.46		
$MnSO_4.4H_2O$ (g/L)	2		
$CuSO_4.5H_2O$ (g/L)	0.5		
$CoSO_4.7H_2O$ (g/L)	0.427		
$FeCl_3.6H_2O$ (g/L)	9.67		
H_3BO_3 (g/L)	0.03		
$NaMoO_4.2H_2O$ (g/L)	0.024		
Volume, V (L)	0.5		

Initials _____
Initials _____

Autoclave the solution according to SOP of ACBE

I. PREINOCULATION

C) Petri plates **Date:** _____

Tick each step when ready

1. Weigh the following compounds and dissolve in R.O. water.

COMPLEX MEDIA FOR PETRI PLATES, CM			
Compound	**Concentration, C**	**To weigh (g): C x WV**	**Weigh (g)**
Tryptone (g/L)	16		
Yeast Extract (g/L)	10		
NaCl (g/L)	5		
Bactoagar (w/v)	0.01		
Chloramphenicol (g/L)	0.025	prepared separately	
Working Volume, WV (L):	0.5		

Initials_____
Initials_____

2. Adjust pH to 6.95 using NaOH solution (4M)

Initial pH: _____

Final pH: _____

Initials_____
Initials_____

3. Divide in two flasks the working volume

Flask 1: _____ (L) Flask 2: _____ (L)

Initials_____
Initials_____

4. Wrap the top of each bottle with cotton wool and aluminium foil nice and tightly

5. Sterilise the 2L flasks in autoclave according to SOP of ACBE, UCL

I. PREINOCULATION

6. Let the bottles to cool down in warm water bath to 45°C

IMPORTANT: Be careful to not let the temperature decrease too much so the solution begins to solidify

7. Add 0.5mL of chloramphenicol solution to one of the 2L flask, using a 0.2μm sterilised syringe filter

8. Let the other 2L flask without chloramphenicol

9. Pour required solution on each Petri plate and label properly

10. Each Petri plate is ready for checking sterility and purity

Initials_____
Initials_____

I. PREINOCULATION

D) Fermentation Ancillaries **Date:**

1. Make up additions of glycerol to increase the biomass, cations solution to increase strength of outer membrane, and additions of lactose to induce the production of Fab antibody fragments

Weigh the following compounds and dissolve in R.O. water.

Compound	Conc, C	To weigh (g): C x WF	Weigh, W (g):	Dilution, (%w/w) D	V_{H2O} (L) (100-D/D)W /1000	V_{H2O} (L)
a) Glycerol 1st shot (g/L)	30	375		80	0.09	
b) Glycerol 2nd shot (g/L)	20	250		80	0.06	
c) Glycerol 3rd shot (g/L)	10	125		80	0.03	
d) Cation solution (mM)		(mM/1000)MW x WF			**1.25W/1000**	
$MgSO_4.7H_2O$	14.4	44.3		----	0.055	
$CaCl_2.6H_2O$	1.7	5.2		----	0.065	
e) Lactose solution (g/L)					**(100-D/D)W /1000**	
1st shot	50	600		50	0.6	
2nd shot	50	600		50	0.6	
3rd shot	50	600		50	0.6	
Working volume, WF (L)	12.5					

IMPORTANT: The lactose shots have to be prepared at the moment of addition.

Initials_____
Initials_____

2. Pour the correct volume of each addition in each ancillary

3. Pour 250mL of PPG in a measuring cylinder

4. Wrap the top of each bottle and the needle of each ancillary with cotton wool and aluminium foil nice and tightly

I. PREINOCULATION

5. Sterilise according to SOP of ACBE, UCL:
   a) Glycerol shots
   b) Cations solution
   c) PPG measuring cylinder
   d) Alkali and acid containers
   e) Sampler
   f) Air filter
   g) $MgSO_4.7H_2O$ solution

6. Prepare alkali and acid solution

a) Prepare alkali solution: 15% ammonia solution (1:1 dilution of 30% ammonia with RO water)

V (L) _____ (30% ammonia solution) V (L) _____ (RO water)

V_{TOTAL} (L) _____

b) Prepare acid solution: 1 in 20 dilution of concentrated sulphuric acid with RO water

V (L) _____ (sulphuric acid) V (L) _____ (RO water)

V_{TOTAL} (L) _____

Initials_____
Initials_____

7. The solutions and the ancillaries are ready

I. PREINOCULATION

E) Complex Media, CM **Date:** _____

Tick each step when ready

1. Weigh the following compounds and dissolve in enough R.O. water.

COMPLEX MEDIA, CM			
Compound	**Concentration, C**	**To weigh (g): C x WV x NF**	**Weigh (g)**
Tryptone (g/L)	16		
Yeast Extract (g/L)	10		
NaCl (g/L)	5		
Chloramphenicol (g/L)	0.025	Prepared separately	
Working Volume, WV (L):	0.25		
Number of Flasks, NF**			

*a minimum of 2 flasks is recommended

Initials_____
Initials_____

2. Adjust to a pH of 6.95 using NaOH solution (4M)

Initial pH: _____

Final pH: _____

Volume used (L): _____

Initials_____
Initials_____

3. Pour in 2L flasks the correct working volume

4. Wrap the top of each bottle with cotton wool and aluminium foil nice and tightly

5. Sterilise the 2L flasks in autoclave according to SOP of ACBE, UCL

6. Let the bottles to cool down

I. PREINOCULATION

7. Add 0.5mL of chloramphenicol solution to each 2L flask, using a 0.2μm sterilised syringe filter

8. Keep the top of each bottle with cotton wool and aluminium foil nice and tightly

9. Each flask is ready for inoculation

I. PREINOCULATION

F) Defined Media, DM (flasks) **Date:** _____

Tick each step when ready

1. Weigh the following compounds and dissolve in enough R.O. water.

DEFINED MEDIA (FLASKS)			
Compound	**Concentration, C**	**To weigh (g): C x WV x NF**	**Weigh (g)**
$(NH_4)_2SO_4$ (g/L)	5		
NaH_2PO_4 (g/L)	2.8		
KCl (g/L)	3.87		
Citric Acid (g/L)	4		
Glycerol (g/L)	30		
Trace Elements (mL/L)	10	Prepared separately	
$MgSO_4.7H_2O$ (g/L)	1	prepared separately to avoid precipitation	
Chloramphenicol (g/L)	0.025	prepared separately	
Working Volume, WV (L):	0.25		
Number of Flasks, NF*			

*a minimum of 5 flasks is recommended

IMPORTANT: Prepare 25mL more of DM for a control flask that will be used for the fermenter

Initials_____
Initials_____

2. Adjust pH to 6.95 using NaOH solution (30g/L to avoid adding excessive volume)

Initial pH: _____

Final pH: _____

Volume used (L): _____

Initials_____
Initials_____

I. PREINOCULATION

3. Pour in 2L flasks the correct working volume of Defined Medium (without $MgSO_4.7H_2O$ solution)

Working Volume (L)	_____
- Volume of Trace Elements (L)	_____
- Volume of $MgSO_4.7H_2O$ (L)	_____
- Volume of Initial Inoculum (10% v/v)	_____
Total Initial Volume (L)	_____

4. Pour 25mL of media in a 250mL Erlenmeyer flask. This is the control to check the media in the fermenter

5. Prepare an empty 250mL Erlenmeyer flask. This is a control to check fermentation conditions

6. Wrap the top of each bottle with cotton wool and aluminium foil nice and tightly

7. Sterilise the flasks in autoclave according to SOP of ACBE, UCL the following items:

8. Let the bottles to cool down

9. Add 2.5mL of $MgSO_4.7H_2O$ solution to each 2L flask

10. Add 2.5mL of Trace elements solution to each 2L flask

11. Add 0.5mL of chloramphenicol solution (already prepared for CM) to each 2L flask, using a 0.2μg sterilized syringe filter

12. Keep the top of each bottle with cotton wool and aluminium foil nice and tightly

13. Each flask is ready for inoculation

Initials_____
Initials_____

I. PREINOCULATION

G) Defined Media (fermenter) **Date:** _____

Tick each step when ready

1. Weigh the following compounds and dissolve initially in 3L of R.O. water.

DEFINED MEDIA (FERMENTER)			
Compound	**Concentration, C**	**To weigh (g): C x WV**	**Weigh (g)**
$(NH_4)_2SO_4$ (g/L)	5		
NaH_2PO_4 (g/L)	2.8		
KCl (g/L)	3.87		
Citric Acid (g/L)	4		
Glycerol (g/L)	30		
$MgSO_4.7H_2O$ (g/L)	1	use the solution prepared for DM(fl)	
Trace Elements (mL/L)	10	prepared separately	
Chloramphenicol (g/L)	0.025	use the solution prepared for CM	
Working Volume, WV (L):	12.5		

Initials_____
Initials_____

2. Add 5L of R.O. water in the vessel of the 20L fermenter

3. Pour DM in the vessel of the 20L fermenter

4. Add R.O. water until a volume of 11L

5. Sterilise the fermenter according to SOP of ACBE, UCL

6. Install al the ancillaries

I. PREINOCULATION

7. Adjust pH to 6.95 using alkali solution

Initial pH: _____

Final pH: _____

Volume used (L): _____

Initials _____
Initials _____

8. Take a sample to check pH

pH: _____

Initials _____
Initials _____

9. Add 125mL of $MgSO_4.7H_2O$ solution

10. Add 125mL of Trace elements solution

11. Add 25mL of chloramphenicol solution using a 0.2μg sterilized syringe filter to the 20L
fermenter.

12. Take a sample to check sterility. Inoculate two Petri plates (with and without chloramphenicol)

13. Check initial volume of fermentation

INITIAL VOLUME (L) OF FERMENTATION	
Volume of Media	11
Volume of $MgSO_4$ solution	0.12
Volume of chloramphenicol	0.022
Initial volume of Fermentation	11.142

14. The fermenter is ready for inoculation

Initials _____
Initials _____

II. INOCULATION

a) Inoculation in Complex Media, CM with a vial from the Working Cell Bank, WCB

Date: _____

Tick each step when ready

1. Take a vial from the WCB and use 0.25mL to inoculate each flask with 0.25L of CM

Date: _____ Time: _____ Run time (hrs): _____

Initials: _____
Initials: _____

2. Measure Optical Density at 600nm of each flask

OD_{600} : _____

Date: _____ Time: _____ Run time (hrs): _____

Initials: _____
Initials: _____

3. Inoculate culture in Defined Media

b) Inoculation in Defined Media (flasks) from the culture in CM Date: _____

1. Inoculate NO MORE than 10% v/v to obtain an initial OD_{600} in Defined Media of 0.3-0.4 on each 2L flask

$$OD_{\text{Defined Media}} \times V_{\text{Defined Media}} = OD_{\text{Complex Media}} \times V_{\text{Complex Media}}$$

2. Measure initial Optical Density at 600nm on each flask

OD_{600} :

_____

Date: _____ Time: _____ Run time (hrs): _____

Initials: _____
Initials: _____

II. INOCULATION

3. Measure Optical Density at 600nm on each flask

OD_{600}

:_____

Date: _____ Time: _____ Run time (hrs): _____

Initials: _____
Initials: _____

4. Take a sample to check purity. Inoculate two Petri plates (with and without chloramphenicol)

5. Check media in the fermenter by inoculating 2.5mL of inoculum into a 250mL Erlenmeyer flask with 25mL of DM (previously prepared).

6. Inoculate fermenter

c) Final inoculation into fermenter from the culture in Defined Media (flasks)

Date: _____

1. Inoculate 1.25L volume (5 flasks) to obtain an initial OD_{600} into the fermenter of 0.2-0.3 (if necessary wait until the OD increase)

$$OD_{\text{Defined Media}} \times V_{\text{Defined Media}} = OD_{\text{Fermenter}} \times V_{\text{Fermenter}}$$

2. Take a sample to check Optical Density at 600nm in the fermenter

OD_{600} : _____

Date: _____ Time: _____ Run time (hrs): _____

Initials: _____
Initials: _____

II. INOCULATION

3. Take a sample to check purity. Inoculate two Petri plates (with and without chloramphenicol)

4. Check fermentation conditions by taking a sample of 25mL and put it into a 250mL Erlenmeyer flask (previously prepared) to continue the growth in a shaker at 30°C

5. Check fermentation working volume

WORKING VOLUME (L) OF FERMENTATION	
Initial volume of Fermentation	11.142
Volume of inoculum	1.25
WORKING VOLUME	12.4

Working Volume (L): _____

Comments/Observations_____
_____
_____

Initials: _____
Initials: _____

III. FERMENTATION

a) Fermentation process **Date:** _____

1. Fermentation starts with the following operation conditions:

a) T= 30°C (when the biomass reaches a reading of 40 OD the temperature is reduced to 27°C to reduce specific growth and promote correct folding of Fab)

b) rpm= 500 (initially, but will vary to maintain a dissolved oxygen tension (DOT) at 30%).

c) Airflow rate= 3Lpm (initially, but will vary to maintain a dissolved oxygen tension (DOT) at 30%).

d) pH= 6.95

2. Collect sample at t=0 and each 4hrs to measure OD and glycerol consumption by HPLC. Fill the Fermentation Run Sheet (FRS).

3. Add the required solutions according to the OD. Fill the Fermentation Run Sheet (FRS).

OPTICAL DENSITY (OD)	ADDITION
15	1st shot of Glycerol
35	2nd shot of Glycerol
40	Cation solution
50	3rd shot of Glycerol and Lactose

NOTE: The first shot of glycerol is expected around the first 22 hrs of fermentation

Further additions of Lactose will be each 5hrs

III. FERMENTATION

4. Check total volume of additions

TOTAL VOLUME (L) OF ADDITIONS			
Addition	Mass, W (g)	Volume,V (L)	Vtotal (L) $W/\delta + V$
a) Glycerol 1st shot	375	0.09	0.39
b) Glycedrol 2nd shot	250	0.06	0.26
c) Glycerol 3rd shot	125	0.03	0.13
d) Cation solution			V_1+V_2
$MgSO_4.7H_2O$	44.3	0.055	
$CaCl_2.6H_2O$	5.2	0.065	0.12
e) Lactose			**1.5V**
1st Lactose shot	625	0.625	0.94
2nd Lactose shot	625	0.625	0.94
3rd Lactose shot	625	0.625	0.94
FINAL VOLUME	3.72		

5. Check final volume of the fermentation

FINAL VOLUME (L) OF FERMENTATION	
Working Volume	12.4
Additions	3.72
FINAL VOLUME	16.12

b) Harvest **Date:** _____

1. Harvest the broth of the fermentation according to SOP of ACBE, UCL.

Final OD: _____
Final Volume (L): _____
Biomass expected*: _____

*NOTE: Assuming 2.6g/L of biomass per unit of OD

2. Clean up the fermenter according to SOP of ACBE, UCL

c) Analysis of samples and results **Date:** _____

1. Start the analysis of samples (ELISA and Dry Cell Weight)

III. FERMENTATION

Final Comments or Observations

Name_____ Signature_____
Name_____ Signature_____

FERMENTATION RUN SHEET

Lot/Run Number: _____ **Fermenter:** _____ **Page:** _____/_____

Run Time (hrs) **Time and Date**				
Agitation (rpm) **Aeration** (lpm)				
DOT (%) **T**				
pH **Pressure** (bar g)				
OD (600nm)				
Sample? (Y/N) **Initials**				
Observations/Comments				

Appendix C

Publications:

García-Arrazola, R., Dawson, P., Buchanan, I., Doyle, B., Fearn, T, Titchener-Hooker, N., Baganz, F. "Evaluation of the effects and interactions of mixing and oxygen transfer on the production of Fab' antibody fragments in a gas blended *E. coli* fermentation". In press, *Bioprocess and Biosystems Engineering Journal*, 2005.

García- Arrazola, R., Siu, S.C., Chan, G., Buchanan, I., Doyle, B., Titchener-Hooker, N., Baganz, F. "Evaluation of pH-stat feeding strategy on the production of Fab' antibody fragments and effect on the recovery step of downstream process in *E. coli* fermentations using gas blending". In press, *Biochemical Engineering Journal*, 2005.

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Evaluation of a pH-stat feeding strategy on the production and recovery of Fab' fragments from *E. coli*

Roeb García-Arrazola, Sun Chau Siu, Gerard Chan, Ian Buchanan, Billy Doyle, Nigel Titchener-Hooker, Frank Baganz*

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Evaluation of the effects and interactions of mixing and oxygen transfer on the production of Fab' antibody fragments in *E. coli* fermentation with gas blending

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Abstract

Fermentations carried out at 450 L and 20 L scale to produce Fab′ antibody fragments indicated a serious problem to control levels of dissolved oxygen in the broth due to the large oxygen demand at high cell densities. Dissolved oxygen tension (DOT) dropped to zero during the induction phase and it was hypothesised that this could limit product formation due to inadequate oxygen supply. A gas blending system at 20 L scale was employed to address this problem and a factorial 2^2 experimental design was executed to evaluate independently the effects and interaction of two main engineering factors: agitation rate and DOT level (both related to mixing and oxygen transfer in the broth) on Fab′ yields. By comparison to the non-gas blending system, results in the gas blending system at same scale showed an increase in the production of Fab′ by 77 % independent of the DOT level when using an agitation rate of 500 rpm level and by 50 % at an agitation rate of 1000 rpm with 30 % DOT. Product localisation in the cell periplasm of > 90% was obtained in all fermentations. Results obtained encourage further studies at 450 L scale initially, to evaluate the potential of gas blending for the industrial production of Fab′ antibody fragments.

1. Introduction

Oxygen availability is very critical for the aerobic growth of *E. coli* in fermentations and, at the same time, difficult to achieve, due to the poor solubility of oxygen [16]. In industrial fed-batch fermentations production often begins with an exponential feed phase of a limiting carbon source, until the limit of adequate oxygen transfer is reached [38].

Gas blending has been proposed as a technique to address the problems of inadequate oxygen supply. Flores, *et. al.*, 1994 [14] reported that this led to increases in product yields for xanthan gums during cultivation of *Xanthomas campestris* where the proportion of high molecular weight polymers was higher as DOT levels were increased. Changes in agitation and shear rate are also linked to oxygen mass transfer rates and consequently it is not always possible to identify the independent effect of oxygen levels on the fermentation, and the levels of metabolite production achieved. For example, *Aurebasidium pullulans* was found to be sensitive to high levels of oxygen independent of the agitation rate, and an increase of polysaccharide yield was possible by maintaining a low level of DOT at 1000 rpm [15].

In another study gas blending was used to separate the effects of DOT from agitation in a fungal fermentation for the production of pneumocandins. In this case the effect of dissolved oxygen was independent of agitation rate within a power range of 2-15kW/m^3, helping to define operating boundary conditions for successful scale up to 19 m^3 [35]. Also, the effect of agitation on the interaction between the extent of mixing in Xanthan fermentation broths and the rate of oxygen transfer was investigated. Findings showed that the biological performance of the culture was independent of agitation rate as long as broth homogeneity could be assured and that critical values of DOT of 6 to 10% were maintained in the production phase [2].

Information on the effect of DOT on recombinant protein production by *E. coli* is relatively scarce and no general rules can be derived. DOT has been shown to have variable effects on the production of different recombinant proteins. For example, Li, *et. al.*, 2002 [28] examined four recombinant strains of *E. coli* for the effects of the dissolved oxygen level on the level of biomass, the plasmid content, and the levels of chloramphenicol acetyltransferase and beta-galactosidase. The optimal dissolved oxygen concentration for the specific activity of recombinant proteins was found to be dependent upon the strain. In another study by De León, *et. al.*, (2003) [11] 1 L bioreactors were used to determine the effect of DOT on the production of penicillin acylase by *E. coli* and maximum activity was obtained at 1% DOT. Another work [6] studied overexpression of a target protein (MspI methylase) in recombinant *E. coli*. Results indicated that under oxygen-deficient conditions, the level of target protein decreased drastically.

This study is concerned with maximising the production titres of antibody fragments through control of DOT levels. The importance of Fab′ and other antibody fragments is based on two properties of particular interest: a) highly specific (involving multiple non-covalent contacts between antigen and antibody based upon complementary in shape and charge) and, b) high binding strengths (with affinities between 10^8 and 10^{11} M^{-1}) [17]. The prospect of being able to exploit these properties and generate molecules of high binding affinity against any chosen target led Paul Ehrlich to predict (in his Croonian Lecture to the Royal Society in 1900) an "age of magic bullets" providing great applications in human therapeutics. At present there are approximately 200 recombinant antibodies that are either approved for use or in a late stage of clinical trials for the treatment of large number of diseases. For example, three different humanised Fab′ fragments are in phase II and III of drug development for cancer, rheumatoid arthritis,

and age-related macular degeneration. Furthermore, a chimeric Fab′ has been approved for prevention of blood clotting and refractory unstable angina [9, 36].

Fed-batch fermentations are preferable to batch operation for the production of antibody fragments since batch methods usually result in low biomass concentrations with reported titres from 40 mg/L [7] to 450 mg/L [25]. On the other hand, fed-batch fermentations using a highly defined medium can result in levels up to 50 g/L of dry cell weight [18] and titres up to 1-2 g/L [10]. Titres as high as 3 g/L using an optimised expression vector under non-limited growth conditions have been reported [20].

This study examines the effect of DOT levels and agitation rates on Fab′ production by *E. coli* at 20 L scale. The principal aim was to design and carry out experiments to test the hypothesis that increased Fab′ titres can be achieved in a fermenter system that is not oxygen limited. The questions tackled by this research have been addressed by comparison of a batch-fed fermentation of an oxygen deprived culture with those growing in environments with constant DOT levels of 30% or 50%. In particular, the effect of DOT and agitation rate on growth kinetics, Fab′ yield and cellular localisation were determined.

Furthermore, the interaction between operating parameters and cell productivity has been evaluated by using statistical design of experiments (DoE). It is hoped that the results will help guide future optimisation studies.

2. Material and Methods

2.1. Strain

E. coli strain W3110 pAC tAC 4D5 Fab′ provided by Celltech Chiroscience Ltd, Slough, UK was used to prepare a working cell bank (WCB) as described elsewhere [8]. The plasmid pAC tAC encoded the light chain and heavy chain Fd′ fragment of the parent antibody under transcriptional control of the *E. coli* tac promoter. Each antibody chain was preceded by the *E. coli* ompA signal sequence to direct expression to the periplasmic space.

2.2. Inoculum development

Cultures of 0.25 mL were first grown in 250 mL of complex media (2xYT) in shake flasks. The starter cultures were incubated for 8 hours in an orbital shaker (200rpm) at 30°C. Then an aliquot was taken and the Optical Density (OD) at 600 nm measured. Samples of 25 mL culture (OD_{600} 2.0-3.0) were transferred to defined media flasks of 250 mL working volume. The flasks were incubated for 12 h in an orbital shaker (200 rpm) at 30°C in order to obtain the required OD_{600} of 1.5-2.0. This provided an inoculum in mid-exponential growth phase, and resulted in an initial OD_{600} of 0.2-0.3 in the 20 L fermenter.

The fermenter had an initial working volume of 12.5 L and contained the same defined medium as the shake flasks. For the pilot plant scale experiments a seed fermenter of 15 L working volume was cultivated in order to obtain the required OD_{600} of 6.0-6.5 before inoculating the 450 L fermenter. The fermenter had an initial working volume of 300 L and contained the same defined medium as the seed fermenter. The preparation of the medium was as previously described [8].

2.3. Fermentation protocol & setup

Fermentations were carried out at 20 L and 450 L scale in computer-controlled bioreactors (Applikon, Schiedam, Holland and Chemap AG, Volketswil, Switzerland, respectively). The 20 L vessel was sterilised using a steam jacket and a holding time of 20 minutes at 121 °C. The pH was measured using a pH probe (Broadley Technologies Ltd, Bedford, UK) and maintained at 6.95 +/- 0.05 by computer-controlled additions of either sulphuric acid (5% v/v) or ammonia (15% v/v). The temperature was maintained at 30 °C +/- 0.5 °C by using a heated jacket. The temperature was reduced to 27 °C before induction in order to promote correct product folding. The agitation rate was set at either 500 rpm or 1000 rpm. DOT was monitored using a DOT probe (Broadley Technologies Ltd, Bedford, UK). Fermenter exit/inlet gas compositions were monitored by mass

spectroscopy (MM8 80 Instrument, VG Gas Analysis Ltd, Middlewich, UK). Data was logged using BioXpert software (Applikon, Schiedam, Holland). The airflow rate was set at 2.5 L/min in all the gas blending experiments. Gas blending was achieved by mixing the inlet air with pure oxygen (BOC Gases, Surrey, UK). The valve releasing oxygen into the inlet stream was set according to a computer control so as to maintain the DOT at 30 % or 50 %. The oxygen flow rate was varied under the control of the PID controller from 0– 2.5 L/min.

Samples of 30 mL of fermentation broth were taken in universal bottles every four to six hours in order to measure OD_{600} and on which additions of glycerol, cation solution (magnesium sulphate and calcium chloride to enhance cell wall strength [40]), and lactose were based (see table 1). Two glycerol additions were made during exponential phase after reaching the maximum specific growth rate and a third addition was made prior to addition of lactose (as detailed in table 1). Lactose acted as the inducer for Fab' formation and resulting in a reduction of the cell growth. This batch-fed feeding strategy was based on the protocol developed by Celltech Chiroscience Ltd. and is reported elsewhere [8, 23]. Assays were carried out on these samples to determine the concentrations of residual substrates, the levels of product formation and the biomass achieved.

INSERT TABLE 1

2.4. Biomass measurement by Optical Density

The growth profile of the strain was followed by optical density measurements of the broth, measured at a wavelength of 600 nm with a spectrophotometer (DU-Spectrophotometer, Beckman Instruments Ltd., High Wycombe, UK). Samples were diluted with RO water (reverse osmosis purified) in order that the absorbance measurement was in the linear range of the instrument i.e. 0-0.8 absorbance units.

2.5. Dry Cell Weight (DCW)

Samples of 1 mL were placed into pre-weighed eppendorfs, and then spun down in a mini-centrifuge (Eppendorf Centrifuge 5415R, Hamburg) at 13,000 rpm for 5 minutes. The supernatant was discarded and the recovered pellets were dried to constant weight in an oven at 100 °C for 24 h. The eppendorfs were then weighed again and the difference between the final weight and the empty eppendorfs taken as the DCW.

2.6. Glycerol, lactose and acetate analysis by HPLC

Glycerol, lactose and acetate concentrations were determined via high pressure liquid chromatography (HPLC). Samples of fermentation broth, 1 mL, were centrifuged at 13,000 rpm for 5 minutes, and the supernatant filtered using a 0.22 μm filter (Sigma-Aldrich, Dorset, UK). Glycerol, lactose, and acetate in each sample were separated with an Aminex HPX-87H column (300 mm x 7.8 mm) on a Summit HPLC system (Dionex Corp CA USA), with 5mM sulphuric acid as the mobile phase. Glycerol and acetate concentration were determined using UV detection at 215 nm and lactose concentration was measured using a RI (refractive index) detector (Dionex Solution GmbH, Germering, Germany). The system was calibrated with known concentrations of glycerol, lactose and acetate each time the assay was performed.

2.7. Fab' concentration measurement by ELISA

Fab' concentration in the supernatant was measured by an Enzyme Linked Immuno Assay (ELISA) following periplasmic lysis as described in Bowering, *et. al.*, 2002 [8]. ELISA plates were coated overnight with HP6045 (a mouse antihuman monoclonal antibody supplied by Celltech Chiroscience, Ltd.,) in phosphate-buffered saline (PBS). After washing 4x with distilled water, serial dilutions of samples and standards were performed on the plate in 100 μL sample/conjugate buffer and left to shake at 250 rpm, and room temperature for 1 h. After washing 4x with dH_2O (distilled water), the

revealing antibody GD12 peroxidase (The Binding Site, Birmingham, UK) was added and diluted 1/1000 in sample/conjugate buffer and left to shake at 250 rpm, and room temperature for 1 h. After washing 4x with dH_2O, tetramethyl benzidine (TMB) substrate was added and the absorbance at 630 nm was recorded using an automated plate reader. The concentration of Fab′ was calculated by comparison with purified Fab′ standards of the appropriate isotype. Assays were run at least in duplicate to ensure reproducible results.

2.8. Total Protein Assay

The Total Protein Assay was performed using a BioRad diagnostic kit (BioRad Laboratories GmbH, München, Germany) according to the manufacturer's instructions. Assays were run at least in duplicate to ensure reproducible results within a range of +/- 4%.

2.9. Experimental design

Fermentations in the two different reactor systems were carried out:

a) Fermentation at 450 L scale without gas blending. A single fermentation was performed to evaluate O_2 levels at pilot scale using off-gas analysis.
b) Fermentation at 20 L scale without gas blending. Experiments were performed in duplicate to evaluate O_2 levels at bench scale based on off-gas analysis.
c) Fermentation at 20 L scale with gas blending. A factorial 2^2 experiment [32] was performed to evaluate effects and interactions of agitation rate and DOT level on Fab′ yields and cell growth. As detailed in table 2, four fermentations with different O_2 transfer environments were set and analysed. All fermentations were carried out in duplicate.

INSERT TABLE 2

Statistical analysis of the results obtained was carried using the data analysis and graphical data presentation program MINITAB (Minitab Ltd., Coventry, UK). An standard analysis of variance (ANOVA) was employed to identify and quantify effect and interactions of agitation rate and DOT level on the production of Fab′ fragments.

3. Results and Discussion

Both the fermentations at 20 L and 450 L scale without gas blending had similar initial K_La values. Overall volumetric mass transfer coefficients (K_La) were estimated using the oxygen-balance technique, as described in Bailey, *et. al.* (1986) [5]. Furthermore, both fermentations were operated under cascade control to maintain a DOT level of 30%. Nevertheless, in both cases the DOT level dropped close to zero before induction. By contrast in fermentations which employed gas blending a constant level of DOT (ca. 30%) was maintained throughout the fermentation (see figure 1).

INSERT FIGURE 1

The 450 L fermentation data were only used to assess whether a 20 fold increase in scale has an effect on the fermentation performance. Results at 450 L scale indicated total Fab′ levels of 75 mg/L, which represents 0.0018 mg of Fab′ L^{-1} g^{-1} of lactose consumed. This value was found to be ~95% less when compared to a total Fab′ yield of 35 mg/L at 20 L scale, which represents 0.055 mg of Fab′ L^{-1} g^{-1} based of utilised.

Results at pilot scale strengthened the hypothesis that O_2 limitation could limit product formation as the culture at this scale observed longer periods with DOT levels near to zero resulting in lower product formation per lactose consumed than that at 20 L scale. It is conceivable that the long period of O_2 depletion prior to induction (ca. 10 h) may have lead to a reduction in viable cells and this contributed to the reduced Fab′ production found compared to that at 20L scale.

This paper focuses on comparison of fermentations with and without gas blending at 20 L scale to allow the effect of gas blending on final Fab′ yield, growth kinetics and cellular localisation of the product to be identified. Furthermore, the effect of mixing and oxygen

transfer on biomass levels and product yields were evaluated using a factorial design 2^2. In table 3 the performances of these five different fermentations are summarised. As can be seen, an increase in the production of Fab′ by 77 % was achieved using a low agitation rate independent of the DOT level. Furthermore, similar biomass yields on glycerol ($Y_{X/glycerol}$ of 0.35 ± 0.01) were observed in all fermentations with and without gas blending, except GB-4 (+ rpm & + DOT) that exhibited reduced biomass and product yields. Results suggest that acetate levels be related to biomass yields as GB-4 observed the highest final acetate level and the lowest maximum biomass yield. On the other hand, specific oxygen uptake rates seem to be related to Fab′ yield as the non-GB and GB-4 observed the lowest specific oxygen uptake rates and the lowest Fab′ yields. The following sections discuss each of these aspects in turn.

INSERT TABLE 3

3.1. Effect of gas blending on biomass levels

A maximum specific growth rate of 0.17 +/- 0.02 h^{-1} was obtained for all fermentations with and without gas blending. In figure 2a, biomass accumulation profiles of fermentations GB-1 (low rpm & low DOT), GB-4 (high rpm & high DOT) and the non-GB fermentation are presented. Fermentations GB-2 and GB-3 exhibited very similar profiles to GB-1 (results not shown). Biomass levels of ca. 30 g/L were obtained in all 20 L fermentations except for that when the most extreme conditions of DOT and agitation speed were used (GB-4). For the fermentation GB-4 (+ rpm & + DOT) the biomass levels produced were 17% lower than the average level of all the other fermentations. Therefore, it is possible that the acetate concentration in GB-4 (see table 3) may have reached an inhibitory level only after induction due to the fact that cell growth and metabolic activity are often influenced by the on-going expression of recombinant proteins within the cell [3]. It has been reported that induction of recombinant protein synthesis in high-cell density cultures can impose an additional metabolic burden, thereby inducing a variety of different stress responses, such as acetate excretion [4, 33].

INSERT FIGURE 2

The lower level of biomass in GB-4 was unexpected and the protein concentration was therefore measured in the supernatant to determine whether the reduced biomass levels could be attributed to increased levels of cell lysis or shear damage under conditions of high agitation and aeration. Results presented in table 3 showed total protein values of 25 to 30 mg/L were obtained in supernatants of all fermentations, effectively ruling out cell lysis as a reason for the differences in biomass levels.

As can be seen in table 3, GB-4 (+ rpm & + DOT) exhibited the highest concentration of acetate at the end of the fermentation. Although cycles of excretion and re-consumption could be expected throughout the fermentation, it is an indication that the acetate levels reached in GB-4 most probably where higher than those of the other fermentations. This may explain the low biomass level achieved as it is known acetic acid leads to growth inhibition of *E. coli* [24]. For example it has been shown that in glucose-feedback-controlled fed-batch fermentations, biomass concentrations up to 30 g/L were produced while accumulating 2 g/L of acetate. When 8 g/L of acetate was accumulated the biomass concentration was less than 10 g/L. The acetate level realised was strain specific [30]. The excretion of acetate under fully aerobic conditions was not expected because it is generally accepted that *E. coli* does not produce acetate when growing on carbon sources such as glycerol [19]. This was explained to be an effect of the restricted substrate uptake and the maximal uptake rate was not suppose to reach the threshold required to trigger acetate excretion [20]. However, a study by Korz *et al.* (1996) [26] has shown that acetate is produced up to 3.3 g/L in high cell density fed batch cultures grown on glycerol. Based on their findings the authors concluded that the critical growth rate with regard to acetate formation is approx. 0.17 h^{-1}. This was supported by the work of Macaloney *et al.* (1997) [31] who also found acetate excretion in fed-batch cultures of *E. coli* grown on glycerol when a critical specific growth rate was exceeded. Since the specific growth rates obtained in all GB and non-GB fermentations are very close to the reported threshold value the excretion of acetic acid during the growth phase is likely.

In order to help to explain our findings, glycerol utilisation profiles were determined. In figure 2b, profiles of fermentations GB-1 (low rpm & low DOT), GB-4 (high rpm & high DOT) and the non-GB fermentation are presented. Fermentations GB-2 and GB-3 exhibited similar profiles to GB-4 and GB-1, respectively (results not shown). Values of

specific glycerol uptake rate could not be estimated due to the substrate oscillations related to the batch-fed feeding strategy. Nevertheless, it is noticeable that a 2-fold higher glycerol level at the time of induction could be seen in the fermentations with high agitation rate (GB-2 & GB-4) compared to the fermentations with low agitation rate (GB-1 & GB-3). This could be the result of a higher glycerol uptake rate due to better mass transfer conditions caused by the higher agitation rate. The difference in agitation rate could have affected the fermentation performance with regard to acetate formation and lactose utilisation and thus could have caused an additional strain on the cells leading to a reduction in glycerol consumption after induction. It has been reported that acetate is produced when carbon flux exceeds the biosynthetic demands of the capacity for energy generation within the cell in a glucose-based system [12, 27, 37]. Therefore, a similar phenomenon in this glycerol-based system could have occurred and the carbon flux was exceeded in GB-2 and GB-4 due to the high mass transfer as a result of the higher agitation rate. On the other hand table 3 shows that the final acetate level in the non-GB fermentation was similar to that observed in GB-2 despite using gas blending. Furthermore, the acetate level in GB-2 was 47 % lower than that in GB-4. This last result suggests that oxygen has a significant effect on acetate formation, as the main difference between GB-2 and GB-4 is the DOT level.

It is known that high concentrations of oxygen can be toxic, highly reactive and can spontaneously cause unwanted oxidation reactions within the cell [29]. On the other hand, levels up to 100% air saturation in *E. coli* fermentations using DOT control have been reported with no inhibitory effect on biomass formation [28]. All fermentations in this work used oxygen levels up to 90% air saturation to maintain a constant DOT of 30 or 50%. Nevertheless, GB-4 (+ rpm & + %DOT) observed the highest concentrations of dissolved oxygen at the highest agitation rate. It has been found that the catabolic activities of *E. coli* are affected by the actual oxygen availability per unit of biomass rather than by the residual dissolved oxygen concentration of the culture [1]. Therefore, as GB-4 was the culture with highest DOT level at the highest agitation rate, it is speculated that toxic concentrations of oxygen in the cell could have been reached in this culture due to high transfer rates of O_2. For example, oxygen enriched *E. coli* batch cultures showed that an increase of oxygen supply decreased both the glucose based biomass yield and maximum specific growth rate in addition to increasing excretion of the by-product acetate [34]. It was only hypothesised that the intermediates produced as a

result of the reduction of an oxygen molecule were themselves toxic. Further work is needed to evaluate whether similar behaviour is observed when glycerol is used as the main C-source and a mechanistic explanation can be proposed.

3.2. Effect of gas blending on Fab' production

In this system, lactose acts as inducer of product formation and therefore a reduction of growth is expected due to the switch of metabolism to product expression leading to a redirection of carbon and energy away from cell growth and towards product formation. Results shown in figure 3 indicate the switch of metabolism after induction based on the pH profiles. A pH increase in the culture broth is likely due to the consumption of amino acids for energy generation and the production of ammonia ions associated with the proton symport for lactose uptake [39]. As cells adapted to lactose metabolism, medium pH dropped again. Fermentation GB-4 observed the latest switch of metabolism (~40 h), possibly due to the effect of the high glycerol concentration on lactose consumption. It has been shown that excessive uptake of carbon sources such as glycerol or lactose can be deleterious to *E. coli* cells as a consequence of the formation of high levels of the toxic compound methylglyoxal in the case of glycerol or due to the collapse of the proton motive force when exposed to high levels of lactose [13, 21]. The deleterious effects of both glycerol and lactose are in agreement with the low levels of Fab' seen in fermentation GB-2 and GB-4 and the high levels of GB-1 and GB-3 (these fermentation observed low levels of glycerol at the point of induction). Nevertheless, fermentation GB-2 observed a similar high level of glycerol at the point of induction but the time of switch of metabolism was similar to that of the other fermentations. This suggests again that the effect of oxygen available to the cell is relevant as the main difference between GB-2 and GB-4 is the DOT level.

INSERT FIGURE 3

After induction, cell growth and metabolic activity are often influenced by the on-going expression of recombinant proteins within the cell [1]. This metabolic activity could be related to respiratory data such as RQ values and specific oxygen uptake rates. For example, values of RQ were in agreement with the switch of metabolism. A switch of RQ

ca. 0.7 to ca. 1.0 was observed at the same time as that of the pH peak in all fermentations. Furthermore, when comparing specific oxygen uptake rates obtained from off-gas analysis during the induction period (see table 3), values of ~12 mmoL/g.h^{-1} for both GB-4 and the non-GB fermentations with similar low Fab' yields were found. By contrast, fermentations GB-1 and GB-3 that both yielded higher Fab' titres observed values of ~45 mmoL/g.h^{-1}. It appears that the higher the oxygen consumption is by the cells the higher are metabolic activity and therefore product formation. Nevertheless, at this point it is not possible to determine what percentage of oxygen is related to product metabolism as opposed to cell maintenance and therefore further work is needed to establish if a direct correlation between specific oxygen uptake rate and product yield exist.

In table 4 the effect of gas blending on product formation is summarised. The Fab' titres were measured at the time of harvest (~46 hrs) for all fermentations. Based on these results, a 77 % increase in Fab' yield was achieved when comparing the gas blending system with low agitation rate with the non-gas blending fermentation. However, it can also be suggested that high agitation rate had a detrimental effect regardless of the DOT level used in the gas blending system. In the case of the fermentations without gas blending the cells ran out of oxygen after 30 hours (i.e. during the induction period). In this case, expression of Fab' possibly occurred under conditions of low oxygen which might have limited product synthesis. In terms of economics, low agitation & low DOT (GB-1) is a less expensive system as it requires less oxygen while obtaining similar levels of Fab' and biomass as compared to low agitation & high DOT (GB-3). This will become an important consideration at large-scale. Furthermore, in all gas blending fermentations, the level of periplasmic product localisation was high, with > 85% being retained in the periplasm.

INSERT TABLE 4

A comparison of the final volumetric Fab' concentrations in Figure 4 shows that conditions of low agitation and low DOT (GB-1) and low agitation and high DOT (GB-3) gave 3 fold higher concentrations of Fab' compared to those of the other fermentations.

INSERT FIGURE 4

3.3. Statistical analysis

DoE methodology (Montgomery, 2001) allowed obtaining further information towards the optimisation of this fermentation process. In table 5 the analysis of variance to evaluate the effects and interactions of DOT and agitation speed on Fab′ production is summarised. It can be observed, that the effect of A (agitation speed) is significant for Fab′ titres as the P value is 0.001. This would imply that there is 95 % probability that agitation rate has a large impact on Fab′ yield when considering experimental error from duplicates. The effect of B (DOT) and its interaction with A appeared to be small relative to the main effect of A and seems to be not statistically significant ($P > 0.01$). Results suggest that from the two engineering factors studied, agitation rate is the main factor to focus on towards optimisation of this fermentation process. Nevertheless, further work is needed to evaluate different levels of agitation rate to study the effect on Fab' yields and by-product formation in more detail.

INSERT TABLE 5

Conclusions

The results reported in this work suggest a fermentation operating strategy that employs gas blending can increase Fab′ titres in bioreactor systems that are not oxygen limited when appropriate mass transfer conditions are achieved. Gas blending was successful in maintaining constant levels of DOT at 20 L scale with a resulting increase in the production of Fab′ of 77 % at an agitation rate of 500 rpm independent of the DOT level, compared to operation at the same scale but without gas blending. In all cases, a level of product localisation in the periplasm of 84 - 93% was also obtained.

An increase in Fab′ yields due to gas blending suggests that protein synthesis is related to oxygen consumption, whereby a constant oxygen supply at an appropriate agitation rate supports higher product titres. Results presented here suggest an alternative approach to increase Fab′ yields. However, further work should be carried out towards improving the feeding strategy partly because the current batch-fed feeding strategy leads to glycerol oscillations that might be limiting Fab′ productivity.

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References

(1) Alexeeva, S., Hellingwerf, K.J., Teixeira de Mattos, M.J. (2002) Quantitative assessment of oxygen availability: perceived aerobiosis and its effect on flux distribution in the respiratory chain of *Escherichia coli*. J. Bacteriol. 184 (5): 1402-6.

(2) Amanullah, A., Tuttiet, B., Nienow, A.W. (1998) Agitator speed and dissolved oxygen effects in Xanthan fermentations. Biotech. Bioeng., Vol. 57 2:198-210.

(3) Andersson, L., Yang, S., Neubauer, P., Enfors, S.-O. (1996) Impact of plasmid presence and induction on cellular response in fed-bacth cultures of Escherichia coli. J. Biotechnol., 46: 255-263.

(4) Andersson, L., Strandberg, L., Enfors, S.O. (1996) Cell segregation and lysis profound effects on the growth of Escherichia coli in high cell density fed batch cultures. Biotechnol. Prog., 12: 190-195.

(5) Bailey, J.E., Ollis, D.F. (1986) Biochemical engineering fundamentals. 2nd edition, McGraw-Hill International editions, Singapore.

(6) Bhattacharya, S. K., Dubey, A. K. (1997) Effects of dissolved oxygen and oxygen mass transfer on overexpression of target gene in recombinant *E. coli*. Enzyme Microb. Technol. 20: 355-360.

(7) Berry, M.J., T.A.K. Wattam, J., Willets, N., Linder, T., De Graaf, T., Hunt, M., Gani, P.J., Davis & P. Porter (1994) Assay and Purification of Fv Fragments in Fermenter Cultures: Design and Evaluation of Generic Binding Reagents. J. Immunol. Methods. 167: 173-182.

(8) Bowering, L.C., Bracewell, D.G., Keshavarz-Moore, E., Hoare, M., Neil, A., Weir, C. (2002) Comparison of techniques for monitoring antibody fragment production in *E. coli* fermentation cultures, Biotechnol. Prog. 18:1431-1438.

(9) Brekke, O.H., Loset, G. A. (2003) New technologies in therapeutic antibody development. Current Opinion Pharmacology, 3:544-550.

(10) Carter, P., Kelly, R.F., Rodriguez, M.L., Snedcor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourrel, J.H., Shepard, H.M., Henner, D. (1992) High level *E. coli* expression and production of a bivalent huminizes antibody fragment. Bio/Technology. 10: 163-167.

(11) De Leon, A., Hernandez, V., Galindo, E., Ramirez, O.T. (2003) Effects of dissolved oxygen tension on the production of recombinant penicillin acylase in *Escherichia coli*. Enzyme and Microb. Tech. 33: 689-697.

(12) El-Mansi, E.M., Holms, W.H. (1989) Control of carbon flux to acetate excretion during growth of Escherichia coli in batch and continuous cultures J. Gen Microbiol. 135 (Pt 11):2875-83.

(13) Ferguson, G.P. Tötemeyer, S., MacLean, M.J., Booth, I.R. (1998) Methylglyoxal production in bacteria: suicide or survival? Arch. Microbiol. 170: 209-219.

(14) Flores, F., Torres, L.G., Galindo, E. (1994) Effect of the dissolved-oxygen tension during cultivation of *Xanthomonas campestris* on the production and quality of Xanthan gum. J. Biotech. Vol. 34 2:165-173.

(15) Gibbs, P.A., Seviour, R.J. (1996) Does agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aurebasidium pullulans* in batch culture? Appl.Microbiol Biotechnol. 46: 503-510.
(16) Gupta, A., Rao, G. (2003) A study of oxygen transfer in shake flasks using a non-invasive oxygen sensor. Biotechnol Bioeng. 5;84(3):351-8.
(17) Harris, B. (1999) Exploiting Antibody-based Technologies to Manage Environmental Pollution. Trends in Biotech. Vol. 17, 7:290-296.
(18) Harrison, J.S., Keshavarz-Moore, E. (1996) Production of Antibody Fragments in *Escherichia coli*. Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences. Vol. 182.
(19) Holms, W.H. (1986) The central metabolic pathways of E. coli relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion to acetate. Curr. Top. Cell. Reg. 28 69-105
(20) Holms, W.H. (1996) Flux analysis and control of the central metabolic pathways in E. coli. Microb. Rev. 19 85-116
(21) Hogema, B.M., Arents, J.C., Baden, R., Postma, P. (1999) Autoregulation of lactose uptake through the LacYpermease by enzyme IIA^{Glc} of the PTS in *Escherichia coli* K12. Molec. Microb. 31: 1825-1833.
(22) Horn, U., Strittmatter, W., Krebber, A., Knupfer, U., Kujau, M., Wenderoth, R., Muller, K., Matzku, S., Pluckthun, A., Riesenberg, D. (1996) High volumetric yields of functional dimeric antibodies miniantibodies in *Escherichia coli*, using and optimized expression vector and high-cell-density fermentation under non-limited growth conditions. Appl. Microbiol. Biotechnol. 46, 524-532.
(23) Humphreys D.P., Carrington, B., Bowering, L.C., Ganesh, R., Sehdev, M., Smith, B.J., King, L.M., Reeks, D.G., Lawson, A., Popplewell, A.G. (2002) A plasmid system for optimization of Fab' production in *Escherichia coli*: importance of balance of heavy chain and light chain synthesis. Protein Expr Purif. 26(2):309-20.
(24) Johnston, W., Cord-Ruwisch, R., Cooney, M.J. (2002) Industrial control of recombinant *E. coli* fed-batch culture: new perspectives on traditional controlled variables. Bioprocess Biosystems Eng. 25: 111-120.
(25) King, D.J., Byron, O.D., Mountain, A., Weir, N., Harvey, A., Lawson, D.G., Proudfoot, K.A., Baldock, D., Harding, E., Yarranton, G.T., Owens, R.J. (1993) Expression, purification and characterisation of B72.3 Fv fragments. Biochem J. 290: 723-729.
(26) Korz, D.J., Rinas, U., Hellmuth, K., Sanders, E. A., Deckwer, W.-D. Simple fed-batch technique for huigh cell density cultivation of *Escherichia coli*. J. Biotech. 39 (1995) 59-65.
(27) Lee, S.Y. High cell-density culture of *Escherichia coli*. Trends Biotechnol., 14 (1996) 98-105.
(28) Li X., Robbins, J.W. Jr., Taylor, K.B. (1992) Effect of the levels of dissolved oxygen on the expression of recombinant proteins in four recombinant *Escherichia coli* strains. J. Ind. Microbiol. 9 (1): 1-9.
(29) Lodish, H., Berk, A., Lawrence Zipursky, S., Matsudaira, P., Baltimore, D., Darnell, J. Molecular cell biology, 4th edition, Freeman and Company, New York, 2000.
(30) Luli, G., Strohl, W.R., (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. App. and Env. Microbiol. Vol. 56, No.4, p. 1004-1011.
(31) Macaloney, G., Hall, J. W., Rollins, M.J., Draper, I., Anderson, K.B., Preston, J., Thompson, B.G., McNeil, B. The utility and performance of near-infra red spectroscopy in simultaneous monitoring of multiple components in a high cell density recombinant *Escherichia coli* production process. Bioproc. Eng. 17 (1997) 157–167
(32) Montgomery, D.C. Design and Analysis of Experiments. 5th edition, John Wiley & Sons, Inc., USA, 2001.
(33) Neubauer, P., Lin, H.Y., Mathiszik, B. (2003) Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in Escherichia coli. Biotech. and Bioeng., 83: 53-64.
(34) O'Bierne, D., Hammer, G. (2000) Oxygen availability and the growth of *Escherichia coli* W3110: A problem exacerbated by scale-up. Bioprocess & Biosystems Eng. 23:487-494.

(35) Pollard, D.J., Kirschner, T.F., Hernandez, D., Hunt, G., Olewinski, R., Salmon, P.M. (2002) Pilot-scale process sensitivity studies for the scale-up of fungal fermentation for the production of *pneumocandis.* Biotech. Bioeng. Vol. 78, 3: 270-279.

(36) Roque, A. C., Lowe, C. R., Taipa, M. (2004) A. Antibodies and genetically engineered related molecules production and purification. Biotechnol. Prog., 20:639-654.

(37) Rocha, I., Ferreira, E. C. (2002) On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant *E. coli. Analytica Chimica Acta* 462: 293-304.

(38) Sanden AM, Prytz I, Tubulekas I, Forberg C, Le H, Hektor A, Neubauer P, Pragai Z, Harwood C, Ward A, Picon A, De Mattos JT, Postma P, Farewell A, Nystrom T, Reeh S, Pedersen S, Larsson G. (2003) Limiting factors in *Escherichia coli* fed-batch production of recombinant proteins. Biotechnol Bioeng. 20; 81(2):158-66.

(39) Straight, J., Parulekar, S., Jansen, N. (1989) Bacterial growth on lactose: an experimental investigation. *Biotechnol. Bioeng.*, Vol: 34: 705-716.

(40) Vaara, M. (1992) Agents that increase the permeability of the outer membrane. Microbiological Reviews, 56 395-411.

Table 1. Protocol of additions to the *E. coli* fermentation at 20L scale.

Table 2. Factorial experiment 2^2 to evaluate the effects and interactions of DOT level and agitation rate on Fab′ yields

Table 3. Effect of agitation rate and DOT level on an *E. coli* fermentation performance with and without gas blending to produce Fab′ at 20 L scale

Table 4. Specific Fab′ titres and the proportion of periplasmic expression as functions of scale and application of gas blending in three different fermentation systems: 20 L without gas blending, 20 L gas blending from run 1 to 4 according to the 2^2 factorial DoE (see table 1).

Table 5. Analysis of variance (ANOVA) to evaluate effects and interactions of DOT and agitation speed on the production of Fab′ at 20 L scale in a gas blending system

Figure 1. DOT profiles of Fab' antibody fragment fermentation in a -◆- 450 L scale with no gas blending and two 20 L scale fermentation — with and ---- without gas blending. The arrow indicates the point of induction.

Figure 2. Effect of gas blending on final biomass levels (a) and glycerol uptake rates (b) in two different fermentation systems at 20 L scale (from left to right): -○- non gas blending, gas blending: -●- GB-1 (-rpm, -DOT), and -■- GB-4 (+rpm, +DOT). Error bars represent standard deviations of duplicate fermentations.

Figure 3. Effect of gas blending on metabolic switch from glycerol to lactose based on pH profiles in two different fermentation systems at 20 L scale with gas blending (GB) and without gas blending (non-GB): a) GB-1 (-rpm, -DOT), b) GB-2 (+rpm, -DOT), c) GB-3 (-rpm, +DOT), d) GB-4 (+rpm, +DOT); and e) non-GB.

Figure 4. Final periplasmic and extracellular Fab' antibody fragment concentration in two different fermentation systems: 20L scale with gas blending according to the 2^2 factorial DoE (see table 1), and 20L scale without gas blending.). Error bars represent standard deviations of duplicate fermentations.

Table 1.

Optical Density OD_{600}	**Addition made**	**Mass, W (g)**	**Volume,V (L)**	**V_{total} (L)**
15	Glycerol (30 g/L)	375	0.09	0.39
35	Glycerol (20 g/L)	250	0.06	0.26
40	Cations solution: ($MgSO_4.7H_2O$ to 14.4 mM & $CaCl_2.6H_2O$ to 1.7 mM)	44.3 5.2	0.055 0.065	0.12
50	Glycerol (10 g/L) & Lactose (50g/L)	125 625	0.03 0.625	0.13 0.94

Table 2.

Exp. #	**A = impeller speed [rpm]**	**B = DOT [%]**
GB-1	-	-
GB-2	+	-
GB-3	-	+
GB-4	+	+

A= - (500rpm), + (1000rpm); B= - (30%), + (50%)

Table 3.

EXP	DCW (g/L)	Extra-cellular protein conc. (mg/L)	Final acetate conc. (g/L)	Fab' conc. (mg/L)	Specific oxygen uptake rate[1] (mmoL $g^{-1}h^{-1}$)	Biomass yield on glycerol $Y_{X/glycerol}$[2] (g/g)	Product yield on lactose $Y_{P/lactose}$[3] (mg/g)
Non-GB	32.0	28.8	2.8	28.3	3.1	0.35	0.57
GB-1	30.7	24.6	2.2	134.0	9.7	0.34	2.68
GB-2	31.0	33.4	2.7	71.2	8.1	0.34	1.40
GB-3	30.6	23.4	1.0	133.5	9.8	0.34	2.67
GB-4	23.5	27.6	5.2	23.7	4.2	0.26	0.47

Total working volume = 12.5 L

1. Maximum OUR after induction was used to estimate the values of specific oxygen uptake rates.

2. A total amount of 1125 g of glycerol was used (including initial concentration plus additions)

3. A total amount of 625 g of lactose was added.

Table 4.

Fermentation	**Fab' titres (mgFab'/gDCW)**			
	Periplasm	Extracellular	Total	% retention in periplasm
20L without gas blending	0.9	0.7	1.6	56
GB-1	4.4	0.6	5.0	88
GB-2	1.9	0.4	2.3	84
GB-3	4.0	0.3	4.4	93
GB-4	1.5	0.1	1.6	92

Table 5. Analysis of variance (ANOVA) to evaluate effects and interactions of DOT and agitation speed on the production of Fab' at 20 L scale in a gas blending system

Factor	**Coefficient**	**Std. Dev.**	**t_3**	**P**
A= Agitation	-42.13	3.64	11.57	0.001
B = DOT	-10.95	3.64	3.01	0.057
A*B	-8.62	3.64	2.37	0.099

where t_3 is the "t" test for the distribution F with 3 degrees of freedom and P is the probability to test

Figure 1.

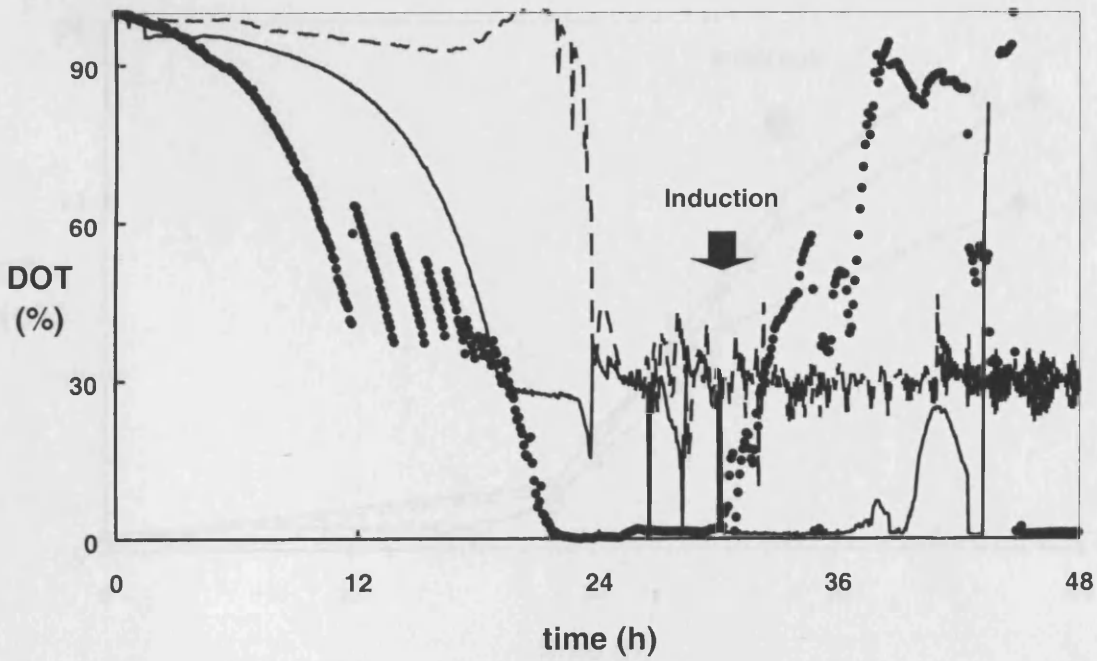
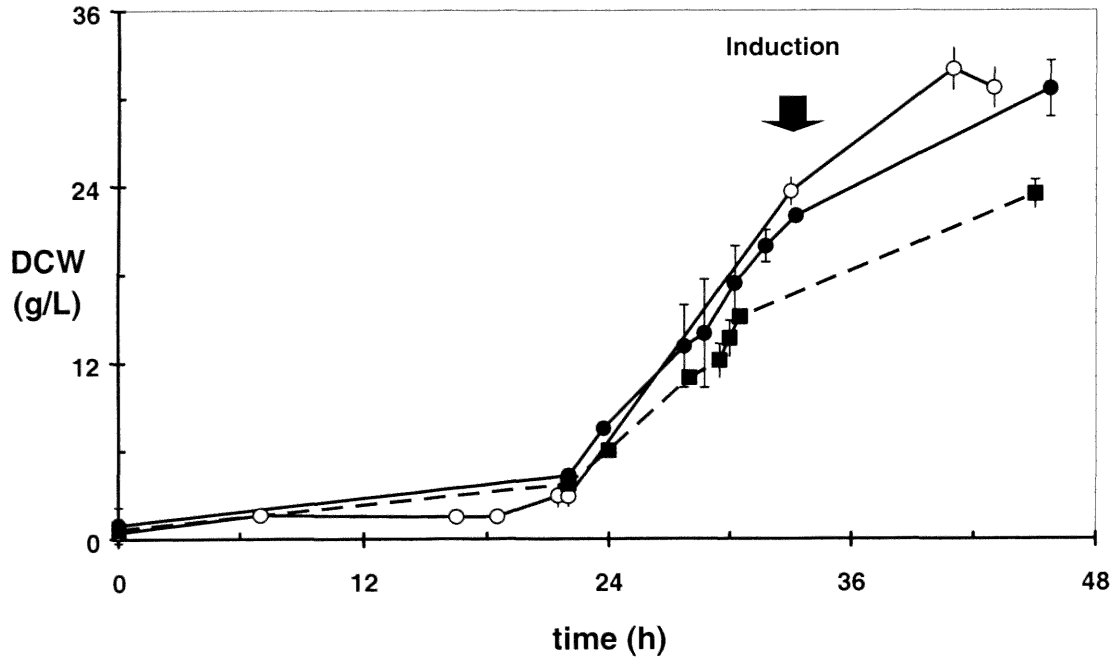


Figure 2.

a)


Induction
DCW (g/L)
36
24
12
0
0
12
24
36
48
time (h)

b)

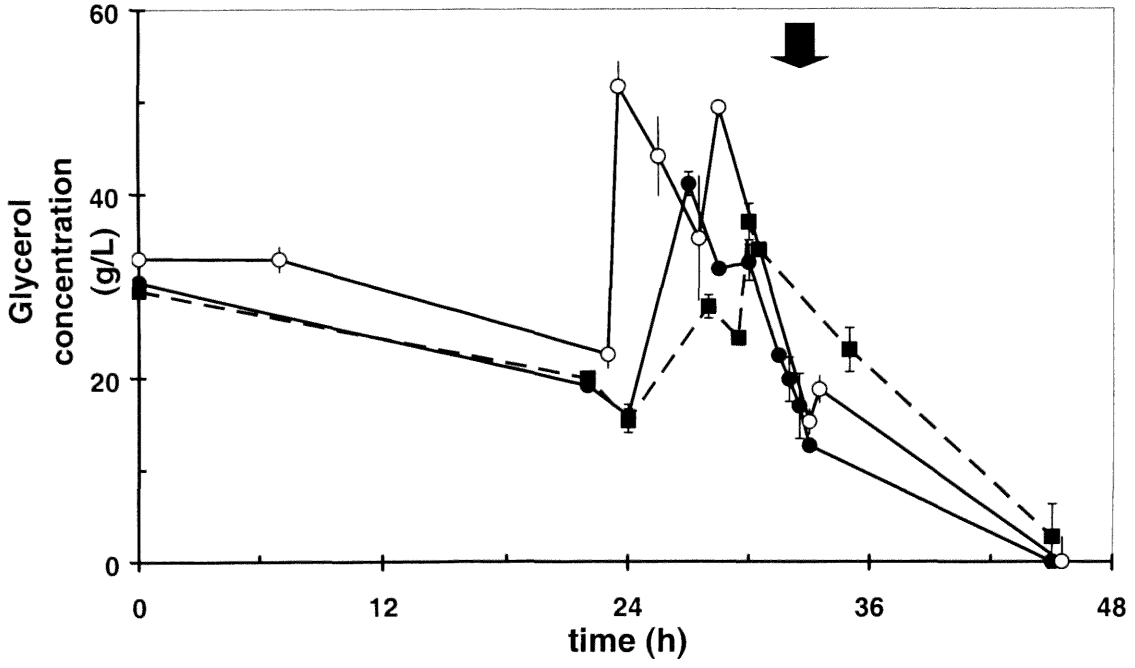

Glycerol concentration (g/L)
60
40
20
0
0
12
24
36
48
time (h)

Figure 3.

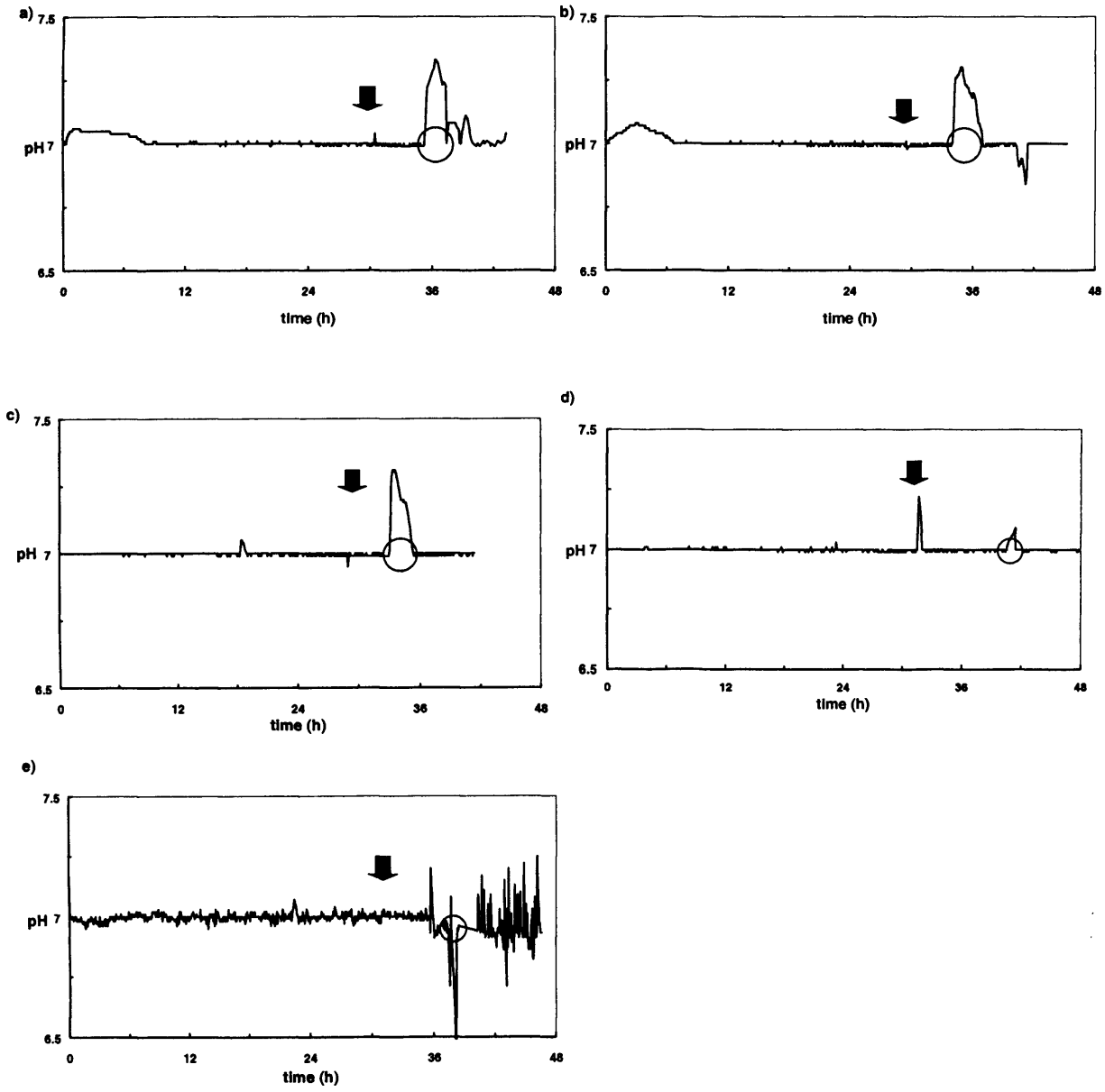
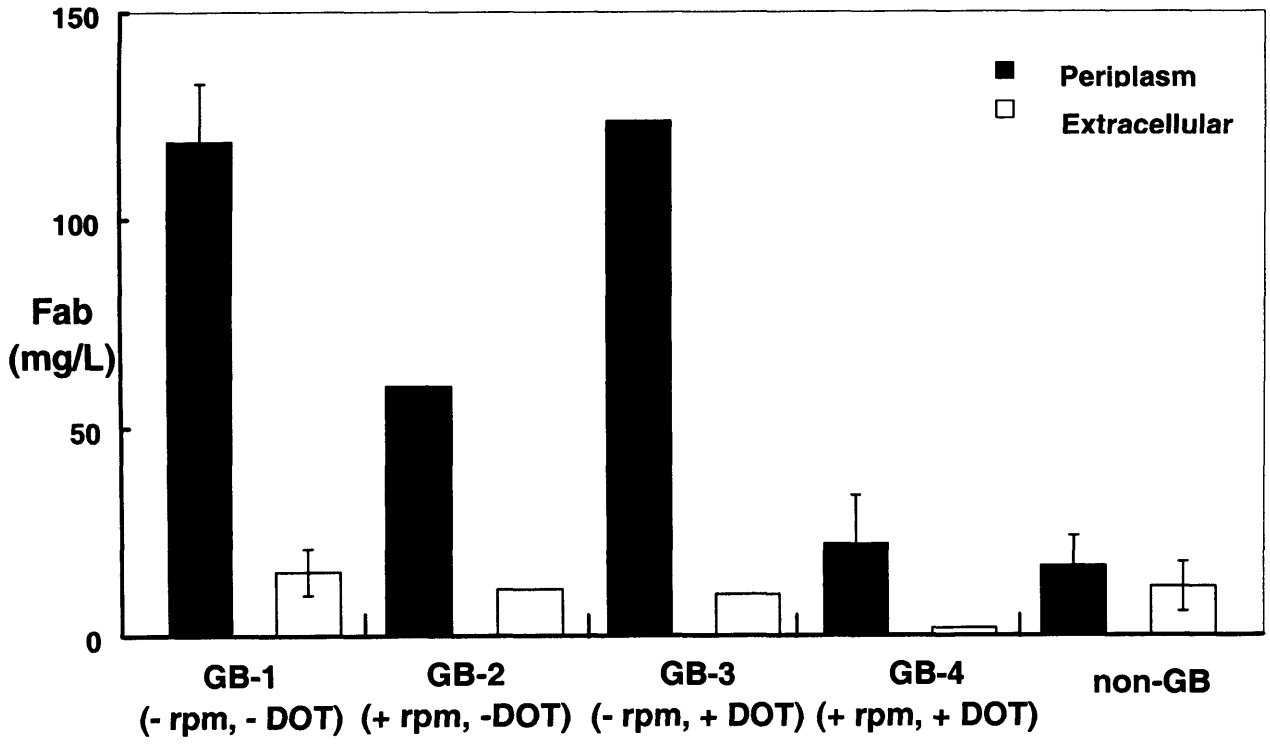

a)
7.5
pH 7
6.5
0
12
24
36
48
time (h)
b)
7.5
pH 7
6.5
0
12
24
36
48
time (h)
c)
7.5
pH 7
6.5
0
12
24
36
48
time (h)
d)
7.5
pH 7
6.5
0
12
24
36
48
time (h)
e)
7.5
pH 7
6.5
0
12
24
36
48
time (h)

Figure 4


150
100
50
0
Fab
(mg/L)
Periplasm
Extracellular
GB-1
(- rpm, - DOT)
GB-2
(+ rpm, -DOT)
GB-3
(- rpm, + DOT)
GB-4
(+ rpm, + DOT)
non-GB

"If you're going through hell, keep going"

Sir Winston Churchill